The Evolutionary Ecology of Transmission Strategies in Protozoan Parasites

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ABSTRACT

In recent years there has been growing interest in applying frameworks from evolutionary ecology to understand infectious disease. It is becoming increasingly apparent that the interactions between parasites within the host environment can shape parasite phenotypes underlying infection dynamics and transmission. However, the spread of the disease will crucially depend on both within-host and between-host dynamics. Bridging these scales is challenging and for vector borne parasites, such as malaria and trypanosomes, will involve gaining a much better understanding of infection dynamics both within the host and vector. I apply evolutionary ecology frameworks including social evolution, life history theory, and phenotypic plasticity to investigate how parasite phenotypes are shaped by within-host and within-vector environments and examine the implications for in-host survival and between-host transmission. Specifically, I demonstrate that;

1. Within the host;
    i. In accordance with theory malaria parasites detect and respond to the presence of competitors by altering reproductive strategies to maximise in-host survival. Furthermore, these strategies are fine tuned in response to variation in the within-host environment, including the availability of resources.
    ii. The reproductive investment strategies of malaria parasites can be applied to explain the transmission strategies of African trypanosomes. This shows how general evolutionary frameworks can be applied to a novel parasite species and demonstrates the explanatory power of an evolutionary approach.
    iii. The complexity of the within-host environment poses specific statistical challenges for examining the temporal dynamics of parasite life history traits that are often not adequately dealt with, potentially leading to type 1 errors. Methods to evaluate levels of autocorrelation and how to deal with it are applied to datasets of within-infection dynamics.
2. Within the vector;
    i. Malaria parasites undergo programmed, apoptotic cell death. The occurrence of, and putative explanation for, apoptosis in protozoan parasites is controversial. I demonstrate the importance of quantitative methods and parasite ecology in testing the evolutionary explanations for parasite apoptosis.
    ii. The links between within-host dynamics and within-vector dynamics are complex and can lead to counter-intuitive implications for the success of between-host transmission. Density-dependent processes result in diverse fitness costs to parasites of crowding. More broadly, these processes could explain why parasites undergo apoptosis.

In general my results demonstrate, across vertebrate hosts and insect vectors, how the interactions between parasites and with their environment shapes traits important for the transmission of infectious disease.

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Declaration

It was only possible to undertake a lot of my research through collaborations, details are provided below. Unless otherwise stated all work was my own.

Chapter 2

The data in this chapter came from an experiment set up by Sarah Reece to examine sex ratios in single and mixed infections. Data was collected by Damien Drew. I performed all the analyses presented here and wrote the subsequent manuscript and chapter.

Chapter 4

The initial simulations in this chapter were performed with help from Nick Colegrave and example data was provided by Sarah Reece and Ricardo Ramiro. I completed the simulations, analysed the data and produced the subsequent manuscript and chapter.

Chapter 5 & 6

The data collected in these chapters was from experiments that I designed and led but with practical assistance from Aidan O'Donnell and honours students Fiona McTaggart and Toby Holland. I analysed all data and produced subsequent manuscripts. The theoretical predictions in appendix 5.10. were formulated by Andy Gardner with input from me on the biological assumptions and background.

This work has not been submitted for any other degree or professional qualification

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CHAPTER 1

GENERAL INTRODUCTION

1.1.Summary

Theory from evolutionary biology and ecology is increasingly being applied to the study of infectious diseases (Altizer *et al.* 2003; Combes 2001; Day 2003; Ewald 1983; Frank 1996; Frank 2002; Galvani 2003; Paul *et al.* 2003; Schrag and Wiener 1995; Stearns and Koella 2008; Williams and Nesse 1991). This approach has not only led to important insights into the biology of disease-causing organisms, but also provided novel tools for addressing more general questions in evolutionary ecology (Buckling *et al.* 2009; Foster 2005; Poulin 2007; Reece *et al.* 2009; West *et al.* 2007; West *et al.* 2006).

A fundamental question in the field of parasite evolutionary ecology is how parasite phenotypes and infection dynamics are shaped by interactions with co-infecting individuals and the within-host environment (Combes 2001). This can be complex as the nature of the interactions that parasites experience will depend both on the characteristics of other individuals sharing the host (e.g. the density and relatedness of co-infecting strains; Brown *et al.* 2002; Foster 2005; Mideo 2009; Read and Taylor 2001; West *et al.* 2006) and the characteristics of the within-host environment (e.g. resource availability and anti-parasitic immune responses; Pedersen and Fenton 2007; Reece *et al.* 2009; Thomas *et al.* 2002). Furthermore, these factors will interact and their relative importance will vary over the course of an infection and between different hosts. This leads to substantial variation in parasite traits and behaviours within and across infections, both within and between genotypes (Poulin 2007). Explaining this variation, and the relative contributions of parasite and host factors to these patterns, is a major challenge. However, general evolutionary theory can be applied to single-celled parasites providing a framework for understanding how and

why parasites alter life-history strategies (Paul *et al.* 2003; Poulin 2007; Reece *et al.* 2009).

Of particular interest are traits underlying harm to hosts (i.e., virulence) and transmission, such as parasite proliferation and investment in transmission stages. However, understanding the impact of these traits at the epidemiological level will involve linking within- and between host processes (Day *et al.* 2011; Mideo *et al.* 2008a; Mideo *et al.* 2011). For vector-borne parasites, such as malaria and trypanosomes, this will mean understanding parasite strategies and infection dynamics both within the vertebrate host and within the insect vector. The work described in this thesis takes theory from life-history evolution, kin selection and ecology and applies them to malaria and trypanosome parasites using techniques from cell biology and parasitology. Using this integrative approach, I have answered specific questions relating to parasite investment in transmission stages within the host and infection dynamics within the vector. Due to the broad nature of the research, detailed introductions are provided for each chapter. Here, I briefly outline the key evolutionary and ecological theory behind the questions my research has addressed and introduce the mains aims of my thesis.

1.2.Life-history theory and phenotypic plasticity

Life history theory lies at the heart of evolutionary biology, with the aim of explaining diverse and complex life-history traits under a simple and unifying explanatory framework (Stearns 1992). Life-history traits are key maturational and reproductive phenotypic components influencing fitness (e.g. age at first reproduction, brood size and growth rate; McNamara and Houston 1996; Stearns 1976). While natural selection will act to maximise fitness through expression of beneficial traits, organisms will also face constraints which limit the possible combinations of traits being expressed at any given time (Roff 1992; Stearns 1992; Stearns and Hoekstra 2000). These trade-offs can take multiple forms but the most intuitive and commonly considered is resource limitation: organisms have limited resources to invest in multiple life-history traits (e.g. reproduction, growth, and

repair) and therefore need to balance their relative investment into each (Roff 1992; Stearns 1992; Williams 1966).

Predicting the optimal resolution to life-history trade-offs is made more complex since the best solution will likely depend on the environment an organism experiences. Further, for many organisms this environment will vary temporally and spatially. Life-history traits can change in response to environmental conditions by two distinct, but not mutually exclusive processes (Roff 1992). First, organisms might be able to produce a range of phenotypic responses matched to different environmental conditions. This process, known as adaptive phenotypic plasticity, is central to understanding the effects of environmental variation on evolution and can be broadly defined as a change in the phenotype of a given genotype in response to environmental cues (Scheiner 1993). Phenotypic plasticity enables organisms to respond rapidly to predictable environmental change in ways that maximize fitness. For example, the freshwater crustacean *Daphnia pulex* produces morphological defences, including neck spines, when exposed to predator cues. However, as these defences are costly to produce they only occur when the *Daphnia* detect predators (Hammill *et al.* 2008). Second, with longer-term environmental changes spanning multiple generations, microevolution can occur where population gene frequencies change due to the fact that individuals best adapted to the new conditions will disproportionably contribute to future generations (Stearns and Hoekstra 2000). For example, when a new high coverage drug treatment is introduced, genes for resistance rapidly spread in the population of parasites targeted, since individual parasites harbouring these genes are more likely to survive drug treatment and be transmitted relative to others (Palumbi 2001).

Plasticity and microevolutionary processes are not mutually exclusive and will both contribute to shaping genotypes and phenotypes (Pigliucci 2005). However, the degree of plasticity in natural populations will vary depending on the variability of the environment organisms have evolved under. Organisms that are likely to encounter a range of environmental conditions are predicted to use plasticity to match their phenotype to changes in their circumstances (Pigliucci 2001). But

maintaining the mechanisms by which to detect, process and respond to environmental cues is costly and organisms also risk measuring inaccurate information or responding with inappropriate phenotypes (Ernande and Dieckmann 2004; Ghalambor *et al.* 2007). Therefore, if environmental changes stabilize, phenotypic plasticity may be replaced by fixed strategies (Pigliucci 2001). The impact of phenotypic plasticity on the evolution of fixed traits is unclear – in theory, it can constrain genetic adaptation (by buffering against fitness loses in the face of environmental change), or facilitate genetic adaptation (by moving phenotypes closer to a new optima which can be fixed) (Ghalambor *et al.* 2007; Lande 2009). However, while interest in the impact of phenotypic plasticity on adaptation in other fields is increasing (e.g. climate change; Chevin *et al.* 2010; Reed *et al.* 2010), the implications for infectious disease evolution in the face of interventions and changing environments are overlooked.

1.3. Resource allocation trade-offs

Trade-offs between life-history traits are likely to be many and diverse, but some of the best studied are those between current and future reproduction, and between reproduction and survival (Stearns 1992). What drives these trade-offs is another key concept of evolutionary biology: that reproduction is costly – organisms do not have infinite resources, nor are resources likely to be available for reproduction across all ages (Stearns 1992; Williams 1966). Like all phenotypic traits, the optimal resolution to the trade-off between investment in survival and investment in reproduction will depend on environmental factors and the state of the organism (McNamara and Houston 1996; McNamara *et al.* 2009). Theory predicts that organisms will invest heavily in reproduction under either very good or exceptionally poor conditions, and will be constrained to investing in survival in intermediate situations (Fischer *et al.* 2009; Mideo and Day 2008). This generates a U-shaped relationship: under extremely good conditions organisms can afford to invest in both survival and reproduction; when organisms experience stressful situations they are constrained to investing in survival in order to ensure future reproduction (reproductive restraint; Fischer *et al.* 2009; McNamara *et al.* 2009; Mideo and Day 2008); when survival

becomes extremely unlikely, the optimal strategy is to switch all investment into reproduction (terminal investment; Charlesworth and Leon 1976; Fischer *et al.* 2009; Williams 1966). Although it is possible to predict the general shape of this relationship (figure 1.1.), the exact shape of the curve is likely to vary depending on the genotype examined and the type of 'stress' experienced.

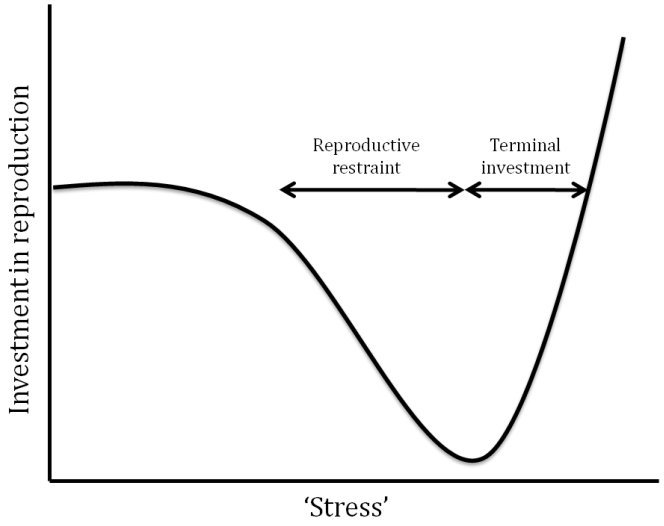

Figure 1.1: Plastic reproductive investment in the face of stress: Illustration of the predicted relative investment in reproduction under increasing stress/deteriorating conditions. Initially as organisms experience stress they are predicted to decrease relative investment in reproduction due to the need to invest in survival. However, once conditions become so bad that survival is unlikely they are predicted to terminally invest into reproduction.

Whilst life-history trade-offs are a key concept for evolutionary ecology, experimental tests of investment decisions and the resulting fitness consequences are challenging. For example, in multi-cellular organisms there will be differential survival of individuals that vary in quality, multiple components of reproductive investment (e.g. egg provisioning, parental care), and decline (through senescence) or improvement (as individuals gain experience) in performance with age. This variation can obscure attempts to examine trade-offs in investment between survival and reproduction and confound the relationship between these investment strategies and lifetime reproductive success (Clutton-Brock 1984). Therefore, despite the

importance for determining evolutionary and ecological processes, strong, direct, empirical support for the adaptive value of life history strategies is lacking.

1.4.Parasite life-histories and social evolution

Whilst the majority of life-history theory was developed for multicellular organisms, single celled parasites face similar challenges and constraints (Poulin 2007). For example, trade-offs may occur between current and future transmission, between the production of different life-stages, or between within-host replication and between-host transmission (Day 2003; Frank 1996). While for metazoans, trade-offs will generally be resolved at the level of the single organism, for parasites a single genotype within an infection can be seen as a comparable target (Gardner and Grafen 2009; West *et al.* 2006b). Therefore, when infections consist of a single parasite genotype (relatedness, r, is equal to 1), trade-offs between life-history traits will be resolved across all individuals within the host in order to maximise transmission over the course of the infection. However, infections will often contain multiple genotypes, and the resolution of parasite life-history strategies and behaviours will be shaped by the presence or absence of co-infecting competitors.

Strategies that are optimal for parasites in single genotype infections may not be optimal for parasites experiencing competition, since selection will maximise their relative fitness rather than their absolute fitness (Chao *et al.* 2000; Mideo 2009). For example, parasites are predicted to exploit host resources 'less prudently' when in mixed infections, due to a so-called 'tragedy of the commons' (Hardin 1968): faster replication provides a benefit to an individual strain (e.g. greater share of resources and higher relative transmission), while the costs (e.g. risk of host death and therefore shortened transmission period) are shared among all strains (Frank 1996). However, predicting the impact of mixed infections on traits of specific parasite species and strains is more complex – mixed infections can lead to either increased or decreased harm to the host depending on the form of competitive interaction and environmental factors, such as parasite density and resource availability (Brown *et al.* 2002; Mideo 2009). Social evolution can help by providing a framework that can

predict the impact of social interactions on parasite traits (West *et al.* 2006b). This approach has received increasing attention, resulting in novel tests and developments for evolutionary theory (e.g. Griffin *et al.* 2004), insights into disease characteristics (Brown *et al.* 2002; Foster 2005; Stearns and Koella 2008), and even possible new intervention strategies (e.g. Brown *et al.* 2009).

1.5. Challenges: within-host environment

For parasites entering a new infection, the within-host environment will vary depending on factors such as host age, infection history and immune status. The characteristics of the within-host environment will also vary temporally with changes in resource availability and mounting immune responses over the course of the infection (Frank 2002; Paul *et al.* 2003). Furthermore, infections are commonly made up of multiple parasite strains or species (Babiker *et al.* 1991; Poulin 2001; Read and Taylor 2001; Vardo and Schall 2007) and interactions between parasites in mixed infections will be complex; interacting with and shaping the within-host environment (Brown *et al.* 2008; Foster 2005; Harrison 2007; Mideo 2009; Read and Taylor 2001). This means that quantifying the influence of different within-host characteristics on parasite life-history traits and dynamics is challenging.

Due to their medical, veterinary and ecological importance, vector borne protozoan parasites (in particular malaria parasites) have attracted considerable research effort, resulting in important insights into diverse aspects of their biology, including their evolutionary ecology (Babiker *et al.* 2008; Drakeley *et al.* 2006; Mackinnon and Marsh 2010; Paul *et al.* 2003; Reece *et al.* 2009). The resultant availability of tools – including model rodent malaria systems, the ability to follow focal genotypes, and the relative wealth of within-host mathematical models – makes malaria parasites an ideal system for studying parasite life-history trade-offs (for malaria life cycle see figure 1.2). However, despite the opportunities this system presents and considerable advancements in tackling some questions, there are still significant gaps in the understanding of traits and processes underlying malaria parasite transmission. For example, malaria parasites experience significant competitive suppression when in

mixed infections, but how this shapes parasite transmission strategies and the impact on transmission of disease is unclear (but see Wargo *et al.* 2007a).

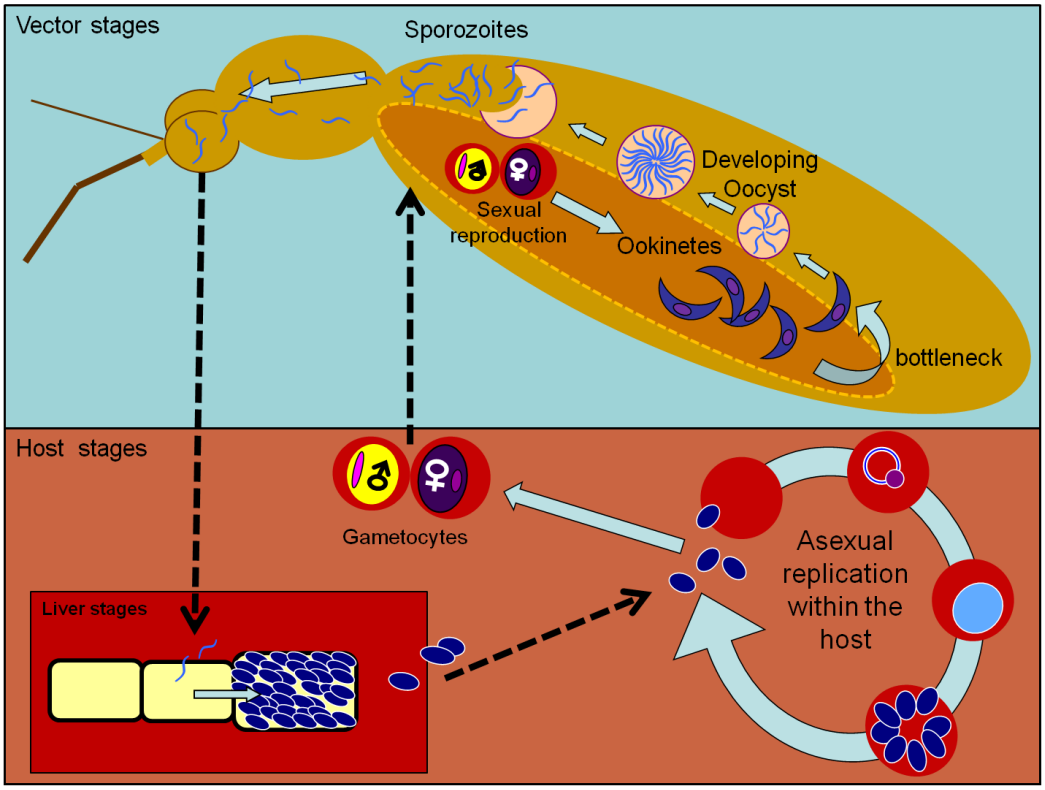

Figure 1.2. Malaria parasite life cycle. While within the vertebrate host malaria infections parasites produce asexual stages, which replicate in red blood cells maintaining the infection, and non-replicating sexual stages (gametocytes), which can be transmitted to vectors. The transmission of malaria parasites through the mosquito, termed sporogony, involves gametocytes within the blood meal rapidly differentiating into male and female gametes and mating (Bannister and Mitchell 2003; Ross 1897). Within 18-20 hours post fertilisation, each zygote transforms into a motile ookinete, which traverses the midgut wall, invades the epithelium of the vector and differentiates into an oocyst. The parasites replicate asexually to produce thousands of sporozoites inside each oocyst before rupturing and releasing sporozoites into the haemocoel. Once sporozoites have migrated to the salivary glands they are ready to be injected into new hosts during the next blood feed (Bannister and Mitchell 2003; Beier 1998). For *P. berghei* in *Anopheles stephensi*, sporogony takes around 21 days (Sinden *et al.* 2007).

While there is a large amount of existing data on infection characteristics (such as parasite density, genetic diversity and the presence and density of transmission stages), it is commonly limited (or reduced) to single trait values per host i.e. either snapshot data (single time point), or cumulative/summary measures (peak parasite density or cumulative production of transmission stages). This can result in important interactions being missed and patterns becoming clouded. For example, in human malaria infections parasite strains circulate and sequester at different times and therefore single samples from infections may dramatically underestimate genetic diversity (Färnert 2008). Analysing infection dynamics is challenging, but advances in statistical techniques can help to examine and control for the relative influence of multiple characteristics of the within-host environment on shaping parasite phenotypes (Paterson and Lello 2003). With the increasing use of such new techniques, it is important to ensure available methods are robust and easily implemented.

1.6. Challenges: between-host transmission

The challenges for studying transmission of infectious disease do not stop at the within-host level. Epidemiology crucially depends on both within-host and between-host processes. Linking across these scales is a key goal for theoretical studies of disease evolution (Alizon *et al.* 2011; Mideo *et al.* 2008a) and promising new tools are becoming available (Day *et al.* 2011; Mideo *et al.* 2011). However, applying these tools to specific disease systems requires the accurate parameterisation of models and detailed data on infection dynamics under varying conditions across both within- and between-host scales is needed. For vector-borne parasites, a further requirement is comprehensive knowledge of parasite interactions and progression through the vector, yet these remain poorly understood.

One challenge for understanding parasite transmission through vectors is mounting evidence that protozoan parasites, including malaria parasites, undergo apoptosis (cell suicide; Al-Olayan *et al.* 2002; Duzensko *et al.* 2006; Hurd and Carter 2004; Pollitt *et al.* 2010; Welburn and Maudlin 1997; Welburn *et al.* 2006). Apoptosis in

protozoan parasites is proposed to be an altruistic trait in order to regulate parasite density (Al-Olayan *et al.* 2002; Duszenko *et al.* 2006; Welburn *et al.* 2006) and has been suggested as a potential target for intervention (Debrabant *et al.* 2003). However, the costs and benefits for parasite fitness remain to be tested. In particular, while the proposed explanations rely on density dependent processes impacting on transmission, examining this is challenging as within-host densities (which are easily measured directly) do not necessarily predict within-vector densities (which are challenging to measure directly). Furthermore, the mechanisms and assays for the detection of apoptosis in protozoan parasites are controversial, and like with many new and emerging fields, technical challenges and debates on terminology have slowed progress (Jimenez-Ruiz *et al.* 2010; Meslin *et al.* 2011; Pollitt *et al.* 2010). Understanding when and why parasites undergo apoptosis will be crucial for linking within– and between-host processes, and before the likely long- and short-term success of any intervention can be evaluated.

Quantifying the shape of the relationship between parasite density in vectors and transmission is a necessary step towards understanding apoptosis, but also an interesting open question in its own right (Basáñez and Ricárdez-Esquinca 2001). In particular, while there is marked variation in the intensity and prevalence of malaria parasites in mosquitoes (Beier *et al.* 1991; Medley *et al.* 1993; Schall 2000; Sinden *et al.* 2007; Tripet *et al.* 2008; Vaughan *et al.* 1994), the reasons for this variation and the implications for patterns of disease transmission remain poorly understood. Furthermore, available data often seem contradictory and the answers to basic questions such as ‘is malaria bad for mosquitoes’ remain uncertain (Ferguson and Read 2002a). Gaining a better understanding of density dependent relationships will have important implications for the transmission of disease (Basáñez and Ricárdez-Esquinca 2001; Combes 2001) and with the current drive to develop transmission blocking interventions, understanding within vector parasite dynamics is of clear importance (Churcher *et al.* 2010).

1.7.Thesis outline and aims

The predictions of evolutionary theory are being met with increasing support across a diverse range of taxa, including single-celled parasites and microbes (Poulin 2007; Reece *et al.* 2009; West *et al.* 2007). The short generation times, ease of experimental manipulation, and the availability of molecular tools mean that parasites provide excellent model organisms for testing evolutionary theory (Buckling *et al.* 2009; West *et al.* 2006). Furthermore, improving understanding of how natural selection shapes the traits of disease-causing organisms will be crucial for predicting the impact of interventions over ecological and evolutionary timescales (Stearns and Koella 2008). However, despite their importance for medicine and evolution, traits underlying disease severity and spread show considerable variation and the proximate and ultimate explanations remain poorly understood. Therefore, this thesis has duel ambitions of using evolutionary ecology to gain insight into disease transmission, and using parasite transmission strategies as a test for the generality of evolutionary theory.

My thesis is conceptually divided into two sections. The first focuses in on the influence of within-host environment on transmission strategies of malaria and trypanosome parasites (chapters 2-4) and the second on malaria parasite dynamics within the vector (chapters 5 & 6). I have added to our understanding of protozoan transmission strategies and developed quantitative methods in parasitology by addressing the following aims:

i. Test recent theory for reproductive restraint by showing how malaria parasites alter their investment into transmission in response to competitors and their within-host environment (chapter 2).
ii. Apply findings from malaria parasite evolutionary ecology to African trypanosomes to show how trypanosomes can provide new opportunities for studying parasite transmission strategies (chapter 3).
iii. Examine how complex within host dynamics pose challenges for statistical inference, and provide solutions for robust analyses (chapter 4).

iv. Investigate the occurrence of, and the evolutionary reasons for, apoptosis in protozoan parasites (chapter 5).

v. Investigate the fitness consequences of high density infections through the effects on vector mortality and parasite proliferation (chapter 6).

CHAPTER 2

COMPETITION AND THE EVOLUTION OF REPRODUCTIVE RESTRAINT IN MALARIA PARASITES

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2.1. Summary

All organisms must trade-off resource allocation between different life processes that determine their survival and reproduction. Malaria parasites replicate asexually in the host but must produce sexual stages to transmit between hosts. As different specialized stages are required for within-host survival and between-host transmisson, the division of resources between these life-history components is a key problem for natural selection to solve. Despite their medical and economic importance, the reproductive strategies of these parasites remain poorly understood and often seem counter-intuitive. Here, I test recent theory predicting that within-host competition shapes how parasites trade-off investment into within-host replication relative to between-host transmission. I show, across several genotypes, that *Plasmodium chabaudi* parasites detect the presence of competing genotypes and facultatively respond by reducing their investment into sexual stages in the manner predicted to maximise their competitive ability. Furthermore, genotypes adjust their allocation to sexual stages in line with the availability of exploitable red blood cell resources. My findings are predicted by evolutionary theory developed to explain life-history trade-offs in more traditionally studied multi-cellular taxa and suggest that the answer to the longstanding question of 'why are so few transmission stages produced' is because in most natural infections, heavy investment in reproduction may compromise within-host survival.

2.2. Introduction

Explaining variation in the life-history traits exhibited by individuals is a major aim in evolutionary biology. Life-history theory provides a solid foundation for understanding plasticity in resource allocation trade-offs and can be used to explain and predict the evolutionary consequences of environmental variation (Roff 1992; Stearns 1992). There is also increasing interest in using an evolutionary approach to understand how parasite life-history traits shape within-infection dynamics and contribute to virulence and transmission (e.g. Brown *et al.* 2002; Day 2003; Eisen and Schall 2000; Foster 2005; Paul *et al.* 2003; Paul and Brey 2003; Reece *et al.* 2009). For organisms such as malaria (*Plasmodium*) parasites, in which within-host replication and between-host transmission are achieved by different specialised stages, the division of resources between these life-history components is a key problem for natural selection to solve. This is analogous to the trade-off between reproduction and maintenance faced by multi-cellular sexually reproducing organisms (Koella and Antia 1995). The assumption that reproduction is costly, resulting in trade-offs between reproduction versus survival and current versus future reproductive effort, is a key concept in evolutionary biology (Williams 1966).

Life history theory has provided a wealth of predictions for how the best solutions to resource allocation trade-offs are influenced by the state of individuals and the environments they experience (Pigliucci 2001; Roff 1992; Stearns 1992; Williams 1966). However, precisely how individuals should adjust their allocation decisions when resources become scarce is unclear. A decrease in the availability of resources can select for increased investment in maintenance (survival), which is achieved by reducing reproductive effort (‘reproductive restraint’; Fischer *et al.* 2009; McNamara *et al.* 2009). Conversely, reduced survival probability can favour increased investment in reproduction (in the extreme, ‘terminal investment’; Charlesworth and Leon 1976; Fischer *et al.* 2009; Williams 1966). These investment decisions lie at opposite ends of a continuum, and whether individuals can adopt the best solution depends on the costs and constraints of plasticity and the accuracy of available information. Testing how individuals trade-off investment between survival and reproduction has been constrained by difficulties in measuring reproductive effort for

multi-cellular organisms (Clutton-Brock 1984), but see (Creighton *et al.* 2009). Reproductive effort can readily be measured for malaria parasites, but despite over a century of research into interventions that block reproduction, their investment strategies remain poorly understood (Reece *et al.* 2009). Previous studies suggest that when within-host survival is threatened, parasites increase investment in between-host transmission at the expense of within-host replication (Bousema *et al.* 2008; Buckling *et al.* 1999a; Buckling *et al.* 1997; Peatey *et al.* 2009; Poulin 2003; Stepniewska *et al.* 2004), but recent evolutionary theory predicts that the opposite should occur (Mideo 2009; Mideo and Day 2008).

As a general rule malaria parasites appear to invest remarkably little in transmission throughout infections (Taylor and Read 1997) and explanations for this apparent reproductive restraint include: reducing virulence experienced by vectors; minimising the extent to which hosts develop transmission blocking immunity; or using numerically dominant asexual stages to shield transmission stages from attack by non-specific immune factors (McKenzie and Bossert 1998; Taylor and Read 1997). However, recent formal theory predicts that within-host competition for resources alone can be sufficient to select for reproductive restraint (Mideo and Day 2008). Genetically mixed infections are common (Babiker *et al.* 1991; Mayxay *et al.* 2004; Vardo and Schall 2007), so parasites frequently interact with co-infecting genotypes (including con- and hetero-specifics). That competition results in suppressed growth and transmission of co-infecting genotypes is well known, but the underlying contributions of exploitation competition (e.g. for red blood cell resources) and apparent (immune-mediated) competition are unclear (Barclay *et al.* 2008; Bell *et al.* 2006; Haydon *et al.* 2003; Mayxay *et al.* 2004; Raberg *et al.* 2006; Taylor *et al.* 1997a). Theory predicts that parasites experiencing local competition (within the host) should divert investment away from reproduction to maximise their ability to replicate and thus exploit the greatest share of red blood cell resources. The recent discovery that malaria parasites can detect and respond to the presence of unrelated competitors (Reece *et al.* 2008) suggests they could also use this information to decide how much to invest in reproduction. Previous attempts to test this prediction have been inconclusive (Bousema *et al.* 2008; Taylor *et al.* 1997b;

Wargo *et al.* 2007a), but these studies did not consider patterns of reproductive effort or performance of focal genotypes in mixed infections.

Here, I use several genotypes of the rodent malaria *Plasmodium chabaudi*, to test whether parasites facultatively respond to in-host competition by decreasing their investment in between-host transmission. I investigate how competition, resource availability, and genetic variation interact to shape patterns of investment in gametocytes during infections. First, I use a bank of genotypes to test for genetic variation in patterns of gametocyte investment throughout infections. Second, I follow three focal genotypes in single and mixed infections with one or more competitors to test whether investment in gametocytes is facultatively reduced in competition. Third, I predict that if reproductive restraint in mixed infections enables parasites to gain the greatest share of exploitable resources, the investment decisions of each genotype will be influenced by the availability of these resources.

2.3. Methods

2.3.1. Infections & experimental design

P. chabaudi genotypes from the WHO Registry of Standard Malaria Parasites (The University of Edinburgh) were used. These wild-type clonal genotypes were isolated from an area where mixed infections are frequent (Carter 1978). Infections described in this paper were originally set up to examine sex ratios in single and mixed infections. Full details are available in Reece *et al.* (2008), but briefly the treatment groups were: (1) six groups of single-genotype infections, consisting of 10^6x AJ, AS, ER, CR, CW or DK parasites; (2) two groups of two-genotype infections, one group with 10^6x AJ+AS parasites and a second group with 10^6x AJ+ER parasites; (3) one group of three-genotype infections with 10^6x AJ+AS+ER parasites. Whilst the starting doses of parasites were higher in mixed infections than single infections, the starting dose of each focal genotype is kept constant. This is necessary because comparing the behaviour of genotypes in different scenarios requires initiating infections of focal genotypes in the same way and only manipulating the in-host environment they experience (i.e. the presence/absence of competition) in each treatment. Previous experiments have demonstrated that variation in the number of

parasites initiating infections has negligible effects on infection dynamics. Specifically, varying starting densities from 1 x 10^2 to 10^8 parasites (for comparison our infections varied from 1 x10^6 to 3 x10^6) had very little impact on asexual production during infections (Timms *et al.* 2001) and more importantly, on gametocyte production (Timms 2001). Thus, our experimental design is standard procedure in studies examining the effect of in-host competition in malaria (including; de Roode *et al.* 2005a; Raberg *et al.* 2006; Reece *et al.* 2008; Wargo *et al.* 2007b) and has also been used for other systems (e.g. Balmer *et al.* 2009; Lohr *et al.* 2010). PCR assays (Drew and Reece 2007) were used to distinguish and quantify the asexual stages and gametocytes produced by each clone throughout infection. These assays enabled following each competitor in the two-genotype infections, which maximised the power of my analyses whilst minimising the number of mice required, but because AS and ER cannot be distinguished only AJ was followed in three-genotype infections.

Mice were 6-8 week male MF1s (in-house supplier, University of Edinburgh) and all treatment groups contained 5 mice. All infections were sampled in the morning so that circulating parasites were in ring or early trophozoite stages and before DNA replication for the production of daughter progeny occurs. I restricted my analysis to data collected between patency of infections (day 5 post infection; PI) and day 12PI. This 1) minimised the time for strain specific immunity to develop (Achtman *et al.* 2007; Quin and Langhorne 2001) that might kill gametocytes and potentially confound estimates of parasite investment decisions, 2) maximised power for the analysis, as 3 mice in the treatment group with the most virulent strain combination (AJ+ER) died on day 12PI, and 3) the effects of competition are strongest during the acute phase (Bell *et al.* 2006). Red blood cell densities were estimated using Flow Cytometry (Beckman Coulter Counter; see Ferguson *et al.* 2003) and reticulocyte densities were estimated from thin blood smears. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.3.2. Gametocyte conversion rate

Competitive suppression in mixed infections is well documented (Bell *et al.* 2006; de Roode *et al.* 2004b; de Roode *et al.* 2005a; de Roode *et al.* 2005b; Paul *et al.* 2002; Raberg *et al.* 2006; Taylor *et al.* 1997a) and results in a reduction in the densities of all competing genotypes (relative to those achieved in single infections). As suppressed genotypes have a smaller pool of parasites from which to produce gametocytes, simply observing lower densities of gametocytes in mixed infections would not reveal whether parasites had invested a lower proportion of their number into developing as sexual stages. Similarly, variation in gametocyte density could be generated from the same relative level of investment by cohorts that simply differ in parasite number. Therefore, I calculated the 'conversion rate' (following Buckling *et al.* 1999a), which is the standard method to measure investment in sexual stages (gametocytes). The conversion rate represents the proportion of parasites in a given synchronous cohort that differentiate into sexual, relative to asexual stages, and takes into account the growth rate of each genotype and the time asexual stages and gametocytes take to mature (24 and 48 hours respectively) in the *P. chabaudi* model system.

2.3.3. Analyses

I used R version 2.6.1 (The R foundation for statistical computing; http://www.R-project.org) for all analyses. I arcsine square root transformed conversion rates and performed analyses using linear mixed effect models with mouse as a random effect to overcome pseudo-replication problems of repeated sampling of infections in each host. I followed model simplification by sequentially dropping the least significant term and comparing the change in deviance with and without the term to χ^2 distributions until the minimal adequate model was reached. Degrees of freedom correspond to the difference of the number of terms in the model. For analyses reported in the 'Resource availability' section, the density of available uninfected red blood cells was calculated by deducting the daily total parasite density from the corresponding daily density of red blood cells.

2.4. Results

2.4.1. Gametocyte investment during infections

I followed six wild type genotypes (AS, AJ, ER, DK, CR and CW) of the rodent malaria *P. chabaudi* to test whether there is within species genetic variation for conversion rates during acute stage infections. Each genotype followed significantly different patterns of conversion across the course of its infections (Genotype ID*Day PI: χ^2_{35}=111.05, p<0.0001; Figure 2.1). Genetic variation in a trait is required for selection to shape its expression and could reflect differences in resource acquisition abilities. Therefore, I tested whether conversion rates related to the abundance and age of available red blood cell resources. The resources available to each parasite cohort explained significant variation in conversion rates, with positive correlations between conversion rate and red blood cell density for five of the six genotypes (RBC*Day PI: χ^2_5=11.73, p=0.039), and the proportion of red blood cells that were reticulocytes (young red blood cells; χ^2_1=4.42, p=0.036) for all genotypes.

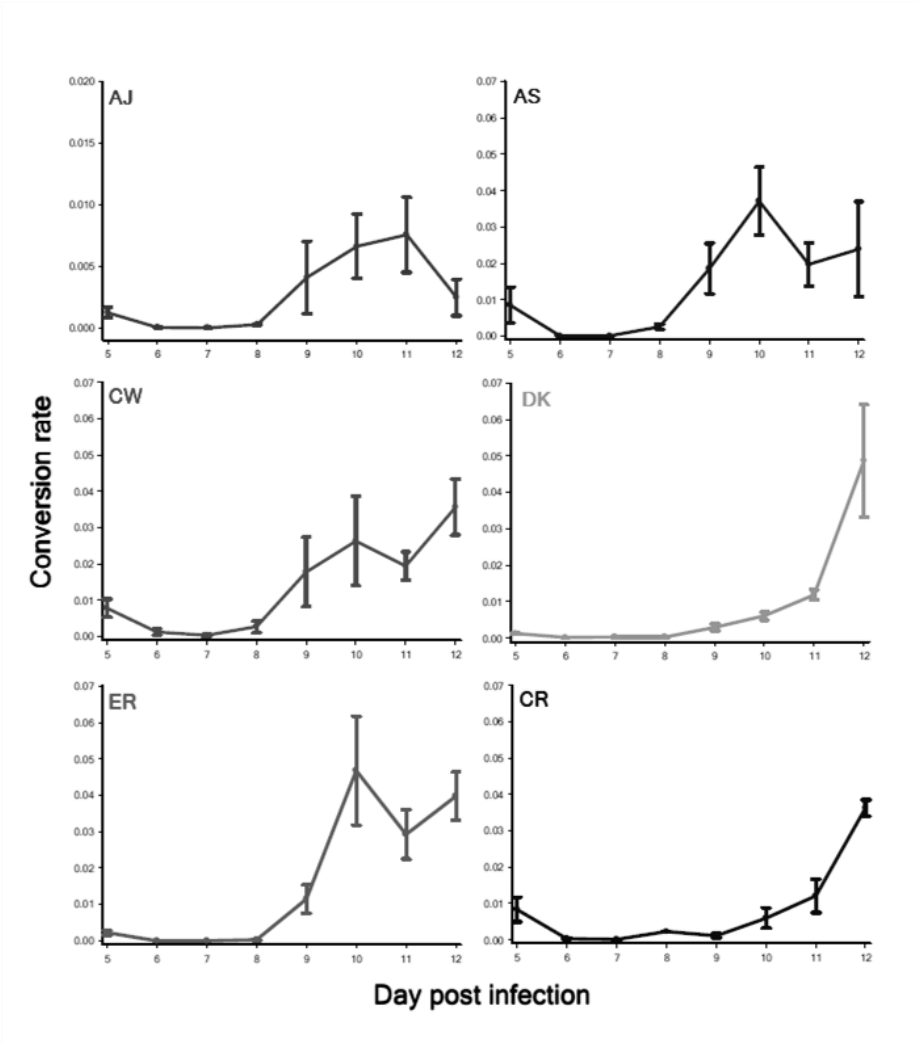

Figure 2.1: Genetic variation for investment into reproduction (transmission stages). Production of gametocytes stages relative to asexual stages (conversion rate) for six wild-type *Plasmodium chabaudi* genotypes over the acute stage of the infection. Error bars show the standard error of the mean for 5 independent infections per genotype.

2.4.2. Effect of competition on gametocyte investment

I used three genotypes (AS, AJ and ER) to test whether conversion rates are adjusted in response to competition. For each genotype, I compared the conversion rates produced in single infections to those produced when in competition with different numbers of unrelated con-specifics. As the quantitative PCR assays could distinguish AJ asexual stages and gametocytes from those of AS and ER, but not between AS and ER (Drew and Reece 2007), I followed AS and ER in double infections (each with AJ) and AJ in double and triple infections (with AS, ER, or both). The conversion rates produced by AJ did not significantly differ depending on the identity (Day*competitor ID: χ^2_8=6.15, p>0.5) or number of co-infecting genotypes (Day*competitor number: χ^2_9=7.78, p>0.5), therefore I simplified my analysis to two treatment conditions of 'alone' or 'in competition' for each of the three focal genotypes.

The existence of competitive suppression is required for our experimental manipulations and the asexual densities of all focal genotypes were significantly reduced in mixed infections (Figure 2.2c; Genotype ID*Day PI*treatment: χ^2_{14}=56.22, p<0.0001). As predicted, when compared to their behaviour in single infections ('alone'), all three genotypes significantly reduced their conversion rates in mixed infections ('in competition') and the magnitude of this effect varied over the course of infections and between the genotypes (Figure 2.2a; Genotype ID*Day PI*treatment: χ^2_{14}=39.59, p<0.0005). Lower gametocyte densities were also observed for each focal genotype in mixed infections (Figure 2.2b; Genotype ID*Day PI *treatment: χ^2_{14}=59.08, p<0.0001). In addition to the effect of competition, conversion rates also had a significant negative correlation with the overall densities of parasites in infections (χ^2_1=10.56, p<0.005), but no significant correlation between conversion rates and red blood cell density or the proportion of red blood cells that were reticulocytes (χ^2_1=0.03, p=0.87 and χ^2_1=2.58, p=0.11 respectively).

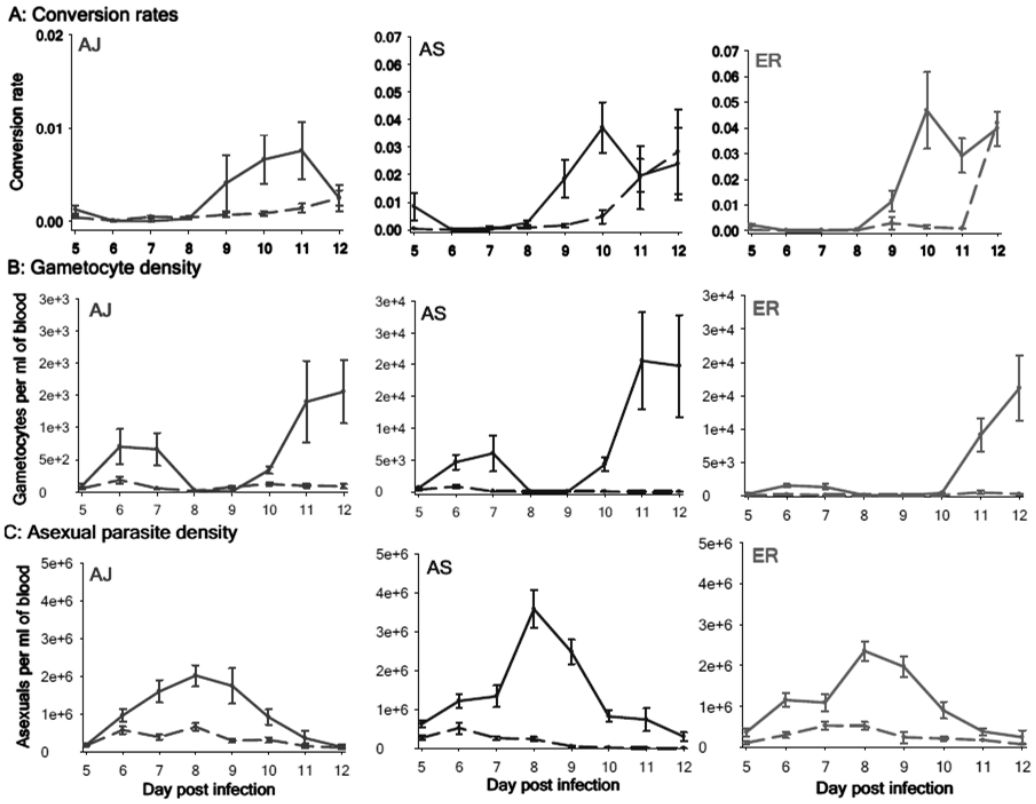

Figure 2.2: Competitive suppression of parasites leads to reduced investment in reproduction (transmission) and lower gametocyte density. Conversion rate (A), Gametocyte density (B) and Asexual parasite density (C) in 3 focal genotypes when alone (solid line) or in competition (broken line). Error bars show the standard error of the mean for between 5 and 15 independent infections per group.

2.4.3. Resource availability

As the single infection data suggests that gametocyte investment is related to the availability of red blood cell resources, but the competition data suggests competition *per se* is more important, I investigated whether resources influence the strategies of competing parasites in more depth. I focussed on the data from mixed infections to test whether the conversion rate of each parasite cohort correlated with the density of available red blood cells. As I found no significant difference in the conversion rates of AJ and ER (χ^2_3=3.66, p>0.1) I simplified my analysis to group these genotypes together. For all genotypes there was a positive correlation between available red blood cell density and conversion rate, and this effect was greater in AS than in AJ/ER (Genotype*resource availability: χ^2_1=12.01, p<0.0005; AS estimated slope = $4.46\text{x}10^{-5}$ ($\pm 1.26\text{x}10^{-5}$); AJ/ER estimated slope = $4.08\text{x}10^{-6}$ ($\pm 5.42\text{x}10^{-7}$).

2.5. Discussion

Testing the assumption that reproduction is costly and trades off against investment in survival is a challenge in evolutionary biology due to potentially confounding effects of differential survival of individuals that vary in quality, multiple components of reproductive investment (e.g. egg provisioning, parental care), decline through senescence, or performance improvement as individuals gain experience. Malaria parasites do not have these complexities and genotypes can be tested in different environments, thus providing a novel system with which to test life history theory (Reece *et al.* 2009). My analyses reveal significant patterns of genetic variation and phenotypic plasticity (Figures 2.1 & 2.2) in the reproductive effort of malaria parasites. My analysis also demonstrates, across several genotypes, that investment in gametocytes is facultatively reduced when parasites experience mixed infections (Figure 2.2) and that this response is influenced by resource availability.

Adopting a reproductive restraint strategy in competition (Figure 2.2) is predicted to enable parasites to maximise their share of exploitable resources in mixed infections (Mideo and Day 2008). Despite the widespread occurrence of genetically mixed-infections in natural populations, how in-host competition has shaped plasticity in parasite life-history trade-offs is rarely considered (but see Wargo *et al.* 2007a). Parasites in mixed infections experience competitive suppression and this could be due to direct (exploitation) competition for resources such as red blood cells (Mideo and Day 2008), or apparent competition in which the presence of another genotype results in cross reactive immune responses that are damaging for both genotypes (McKenzie and Bossert 1998). Diverting investment away from gametocytes and into asexual replication, as observed in this experiment, could benefit parasites experiencing both types of competition. The 'safety in numbers' afforded by reproductive restraint may enable parasites to withstand attack from cross-reacting immune responses as well as maximising the ability to compete for resources. Interactions between these different forms of competition are well known in predator-prey ecology; the presence of a competitor can both reduce food availability and increase predator numbers (Holt 1977; Jones *et al.* 2009). Disentangling the relative influences of exploitation and apparent competition is challenging. This

experiment was not designed to test for apparent competition, but the mixed infections data suggest that exploitation competition plays a role as conversion rates are positively correlated to the availability of red blood cell resources.

Reducing investment in gametocytes as a response to competition is predicted to maximise competitive ability, but the parasites in this experiment – like all other mixed infection experiments with this model system – still experienced suppression (Figure 2.2c). Does this suggest the parasites did not benefit from adopting reproductive restraint, or would they have been even more suppressed without this strategy? Quantifying the effects of reproductive restraint on competitive ability requires comparing the extent of suppression experienced by parasites that can and cannot reduce their gametocyte investment. Measuring the fitness consequences of investment decisions is difficult, but one way to achieve this would be through identifying the mechanism used to detect co-infecting competitors and manipulating parasites to be unable to respond. The costs and benefits of altering gametocyte investment will also depend upon aspects of parasite ecology and population biology. For example, parasites restricted to infecting a specific age class of red blood cells may face more severe resource limitation in competition than more generalist parasites (Reece *et al.* 2005). Resource limitation may occur during periods of anaemia, but for parasites that can infect reticulocytes, anaemia may signal an imminent influx of resources, making reproductive restraint the best short-term solution. Another important aspect of the trade-off between current and future reproduction concerns transmission opportunities to mosquitoes and the type of hosts that parasites will be transmitted to (Alizon and van Baalen 2008; Day 2002; Reece *et al.* 2009). For example, within-host survival may be sufficiently important to constrain gametocyte investment to low levels in areas with irregular or low transmission, or if there is a high probability that the next host already harbours competing parasites (Vardo *et al.* 2007).

In contrast to my data, most previous studies have demonstrated that parasites do not adopt reproductive restraint when experiencing stress. For example, parasites are known to produce more gametocytes when exposed to sub-curative doses of anti-malarial drugs, anaemia, or changes in the age of available red blood cells (Buckling

et al. 1999a; Buckling *et al.* 1999b; Buckling *et al.* 1997; Reece *et al.* 2005; Trager and Gill 1992). Most explanations assume that these changes in the within-host environment cause sufficient reduction in parasite survival to induce terminal investment in reproduction (Buckling *et al.* 1999a; Buckling *et al.* 1999b; Buckling *et al.* 1997; Peatey *et al.* 2009; Stepniewska *et al.* 2004). However, reproductive restraint has now been observed in response to low doses of drugs (Reece *et al.* 2010) and the influx of reticulocytes during anaemia may benefit parasites that can use this resource (Reece *et al.* 2005). Therefore, I suggest that changes in the within-host environment should be evaluated in the context of parasite ecology to interpret whether changes in gametocyte investment are due to reproductive restraint, terminal investment, or a response to increased resources. More broadly, the ability of parasites to fine tune gametocyte investment in response to subtle changes in their in-host environment highlights the importance of measuring and accounting for variation in the these parameters when investigating the effects of experimental manipulations on parasite behaviour.

Few studies (but see Buckling *et al.* 1999a; Buckling *et al.* 1999b; Wargo *et al.* 2007a) have formally tested whether genetic variation in patterns of gametocyte densities are due to different investment strategies or are simply by-products of variation in asexual stage densities. By using a bank of six genotypes and following infections initiated with the same starting dose in immunologically naïve hosts, I have demonstrated genetic variation in reproductive investment (Figure 2.1). The explanations for this variation are not yet known; investment patterns are not related to the virulence rankings of the genotypes (Mackinnon and Read 1999) or competitive ability (Bell *et al.* 2006). Alternatively, there may be genetic variation for the range of red blood ages that parasites can invade and utilise (Antia *et al.* 2008; Mideo *et al.* 2008b). However, whilst my data reveal that gametocyte investment is positively correlated with the abundance of mature and immature red blood cells we did not find any GxE interactions for this pattern. As, in general, *P. chabaudi* are able to infect all ages of circulating red blood cells, all genotypes may simply be able to afford greater investment in gametocytes when resources are abundant (Reece *et al.* 2005).

To investigate whether resource availability matters in competition I focussed only on the mixed infections and asked whether conversion rates of each cohort correlated with the density of uninfected red blood cells. I predicted that gametocyte investment would be positively correlated with available resources. All three genotypes (AS, AJ and ER) supported this prediction, and demonstrate genetic variation in these patterns because the slope for this correlation is greater for AS than for AJ and ER. Why this genetic variation exists is unclear, but could be related to competitive ability as AJ and ER are known to be superior competitors to AS (Bell *et al.* 2006). This could be tested by initiating infections with competing genotypes at different densities and frequencies in hosts with manipulated levels of anaemia to span a broader range of competitive suppression and resource availability. Such experiments, ideally combined with modelling, could also reveal whether decisions span the continuum from reproductive restraint to terminal investment and how parasites respond to different stresses that impact on proliferation rate.

Explaining how parasites respond to changes in their within-host environment is important for understanding patterns of virulence and transmission. All else being equal, when parasites decrease investment in gametocytes, relatively more of the host-damaging asexual stages are produced. Thus, by most measures, a strain that shifts investment away from gametocytes is likely to be more 'virulent' (Mideo and Day 2008). In light of this, my results are also in agreement with a large body of theory on virulence evolution predicting that in multiple infections, parasites are selected to become less prudent (Frank 1994; Frank 1996). Furthermore, if medically relevant parasite species adopt reproductive restraint in response to in-host competition, selection could be strong enough to fix parasite investment in gametocytes at a low level where mixed infections are common. If so, these parasites would have a greater capacity to cause disease when released from competition, for example, as a result of evolving drug resistance and drug treatment removing sensitive competitors (de Roode *et al.* 2004a; Wargo *et al.* 2007b).

Translating the results from a mouse model system to human malaria parasites, especially *P. falciparum,* may be complicated by differences in the traits that underlie virulence. For example, in contrast to *P. chabaudi,* rosetting is a virulence

trait in *P. falciparum*, but occurance of this phenotype also correlates with parasitemia (Rowe *et al.* 2002). If *P. falciparum* responds to competition in the same way as *P. chabaudi*, hosts would be expected to harbour infections made up of fewer gametocytes relative to asexual parasites in endemic regions where mixed infections are frequent. There is some evidence that gametocyte densities of *P. falciparum* are lower in endemic areas and in older individuals (Drakeley *et al.* 2006). A proposed explanation for these trends is that immunity develops more rapidly to gametocytes than asexuals and stronger responses are developed in endemic areas and older patients. However, I suggest that a possible alternative explanation is infections in endemic areas and older hosts are more likely to contain a mixture of genotypes. Now that the tools are available to measure genetic diversity in natural infections it should be possible to test these hypotheses.

Reciprocal competitive suppression is well known in the rodent malaria system (Bell *et al.* 2006; de Roode *et al.* 2004b; de Roode *et al.* 2005a; de Roode *et al.* 2005b; Paul *et al.* 2002; Raberg *et al.* 2006; Taylor *et al.* 1997a) but interactions between co-infecting clones may be more complex in *P. falciparum.* Parasite interactions may span from facilitation (e.g. via changes in the age structure of red blood cells; McQueen and McKenzie 2006), to no effect on each other, to direct competition for resources (e.g. red blood cells; de Roode *et al.* 2005a) and these interactions can be intensified or alleviated by cross reactive immune responses (Barclay *et al.* 2008; de Roode *et al.* 2005a; Gilbert *et al.* 1998; Gupta *et al.* 1994). For example, molecular data suggest that proportions of different clones within mixed infections can fluctuate over time suggesting that multiple clones are not always present in the circulation (Babiker *et al.* 1998). This may also mean that whether a clone appears to be a good or a poor competitor is context dependent. Understanding these interactions in natural settings is a huge challenge but given that there is much variation in the population ecology of human malaria parasites, they offer an attractive system for the study of mixed infections.

In conclusion, the gametocyte investment patterns I observed are predicted by evolutionary theory for life histories and demonstrate that parasites alter their investment in within-host replication (survival) relative to between-host transmission

(reproduction) in response to competition. That resource abundance also mediates this trade-off adds considerable support to the hypothesis that parasites evaluate their social and within-host environments and respond adaptively (Reece *et al.* 2009). My findings also suggest that the answer to the longstanding question of 'why so few transmission stages' (Taylor and Read 1997) is that in most natural infections, the importance of investing in within-host survival constrains parasites to low investment in reproduction. The extent to which individuals display phenotypic plasticity in their reproductive effort depends on the fitness benefits of different life history strategies, the costs and limits of assessing the relevant environmental or internal parameters, and constraints on the range of investment strategies that can be adopted (DeWitt *et al.* 1998). If the proximate mechanisms underlying phenotypic plasticity in malaria parasites can be identified, it may be possible to manipulate their behaviour in clinically and epidemiologically beneficial ways.

2.6. Appendix

After publication of the findings in this chapter I became aware of a potential issue for the analysis of within-host dynamics. Specifically, the analysis of temporal data can be complicated due to the potential for autocorrelation in the residuals of statistical model (see chapter 4). To investigate whether autocorrelation has consequences for the analysis and interpretations of the data presented in this chapter, I checked for autocorrelation in the residuals of minimal models and repeated the analyses fitting an auto-regressive correlation structure (AR1) with day as a time covariate and mouse as a grouping factor (Zuur *et al.* 2009). This allowed me to control for correlation between model residuals in measurements of individual mice on consecutive days in the models. I do this for two of the analyses presented in this paper - 'gametocyte investment during infections' and 'effect of competition on gametocyte investment'. The analysis on 'resource availability' did not involve examining patterns over time and therefore is unaffected by temporal autocorrelation. Below, I present the results of the new analyses and the implications for each question,

2.6.1. Gametocyte investment during infections

The analysis for gametocyte investment during infection (2.4.1) was repeated with the models specified identically to the original analysis apart from the addition of the AR1 error structure. The estimated temporal autocorrelation in my minimal model was 0.38 (i.e. residuals on adjacent days are correlated by 38%). However, repeating this analysis with an AR1 correction had very little qualitative impact on the findings. There was still significant genetic variation for the patterns of conversion rates over infection and a positive relationship between conversion rates and the proportion of red blood cells that were reticulocytes. While there was no longer a significant interaction between genotype and red blood cell density (marginal in the original analysis) there was a significant main effect of red blood cell density (Table 2.1.).

	Predictor variable		p
Original analysis	Genotype*day	χ^2_{35}=114.10	p<0.0001
	Proportion of red blood cells reticulocytes	χ^2_1=4.42	p=0.036
	Genotype*available red blood cell density	**χ^2_5=11.73**	**p=0.039**
With AR1 error structure	Genotype*day	χ^2_{35}=101.33	p<0.0001
	Proportion of red blood cells reticulocytes	χ^2_1= 13.81	P = 0.001
	Genotype*available red blood cells	**χ^2_5=6.31**	**P=0.28**
	available red blood cell density	χ^2_1= 10.65	P = 0.001

Table 2.1. Analyses of investment into gametocytes during single genotype infections with and without the AR1 error structure. Effects where the statistical inference was altered are highlighted in bold.

2.6.2. Effect of competition on gametocyte investment

A limitation of the AR1 error structure is that it is not possible to fit multiple values for the same individual infection on each day. Therefore, as my experimental design made the full use of infection data, which involved tracking all co-infecting genotypes in all mixed infections, it was not possible to exactly repeat the analyses for 'the effect of competition on gametocyte investment' (2.4.2) because this required fitting the three way interaction (Genotype ID*Day PI*treatment). Instead I analysed each of the three focal genotypes separately and tested the statistical significance of all the terms in the original model, both with and without the AR1 error structure. None of the statistically significant results presented in chapter 2 and the resulting publication appear to be type 1 errors as the main effect of competitor*day remained highly significant (p<0.0001) for all analyses. The only slight change in statistical inference was that the borderline non-significant positive correlation between the proportion of young red blood cell (reticulocytes) and the conversion rates of AS becoming borderline significant (Table 2.2.).

Gametocyte investment during infections			
AJ (without AR1)	Variation in response depending on		
	1. Number of competitors?	χ^2_8=8.44	P=0.39
	2. Identity of competitor?	χ^2_{16}=7.73	P=0.46
	Proportion of RBCs reticulocytes	χ^2_1=0.65	P=0.42
	RBC density	χ^2_1=5.81	P=0.016
	Total parasite density	χ^2_1=18.67	P<0.0001
	Competition * day	χ^2_7=52.00	P<0.0001
AJ (with AR1, Phi=0.39)	Variation in response depending on		
	3. Number of competitors?	χ^2_8=7.95	P=0.44
	4. Identity of competitor?	χ^2_{16}=10.29	P=0.25
	Proportion of RBCs reticulocytes	χ^2_1=0.38	P=0.54
	RBC density	χ^2_1=4.17	P=0.041
	Total parasite density	χ^2_1=13.24	P=0.0003
	Competition * day	χ^2_7=40.34	P<0.0001
ER (without AR1)	Proportion of RBCs reticulocytes	χ^2_1=3.25	P=0.07
	RBC density	χ^2_1=0.58	P=0.81
	Total parasite density	χ^2_1=17.04	P<0.0001
	Competition * day	χ^2_6=49.81	P<0.0001
ER (with AR1, Phi=0.06)	Proportion of RBCs reticulocytes	χ^2_1=1.39	P=0.24
	RBC density	χ^2_1=0.20	P=0.65
	Total parasite density	χ^2_1=9.85	P=0.0017
	Competition * day	χ^2_7=54.91	P<0.0001
AS (without AR1)	**Proportion of RBCs reticulocytes**	**χ^2_1=3.02**	**P=0.082**
	RBC density	χ^2_1=0.08	P=0.77
	Total parasite density	χ^2_1=0.08	P=0.78
	Competition * day	χ^2_7=37.12	P<0.0001
AS (with AR1, Phi=0.65)	**Proportion of RBCs reticulocytes**	**χ^2_1=6.22**	**P=0.013**
	RBC density	χ^2_1=0.15	P=0.70
	Total parasite density	χ^2_1=1.42	P=0.2
	Competition * day	χ^2_7=38.83	P<0.0001
Competitive suppression in mixed infections			
AJ	(without AR1)	χ^2_7=72.20	P<0.0001
	(with AR1, Phi = 0.32)	χ^2_7=54.74	P<0.0001
ER	(without AR1)	χ^2_7=57.57	P<0.0001
	(with AR1, Phi = 0.32)	χ^2_7=54.35	P<0.0001
AS	(without AR1)	χ^2_7=89.82	P<0.0001
	(with AR1, Phi = 0.45)	χ^2_7=84.51	P<0.0001
Reduction in gametocytes in mixed infections			
AJ	(without AR1)	χ^2_7=57.22	P<0.0001
	(with AR1, Phi = 0.23)	χ^2_7=51.58	P<0.0001
ER	(without AR1)	χ^2_7=35.40	P<0.0001
	(with AR1, Phi = 0.75)	χ^2_7=29.91	P<0.0005
AS	(without AR1)	χ^2_7=29.60	P<0.0001
	(with AR1, Phi = 0.35)	χ^2_7=23.37	P<0.0001

Table 2.2. Analyses of investment into gametocytes, asexual density and gametocyte density in single and mixed infections with and without the AR1 error structure. Phi values correspond to the estimated level of autocorrelation in the minimal model and effects where the statistical inference was altered are highlighted in bold.

2.6.3. Outcome of re-analysis

While I found evidence of autocorrelation in my models, and while fitting an auto-regressive error structure improved the fit of the model, this did not qualitatively change the key findings presented in chapter 2 and the subsequent publication (Pollitt *et al.* 2011b).

My original analysis suggested a GxE effect for the response to red blood cell availability in the single infections. The new analysis does not support this and instead found that all genotypes responded to increasing availability of red blood cells in the same way by increasing investment in transmission (the same pattern found for five of the six genotypes in the original analyses). In the analysis concerning 'Effect of competition on gametocyte investment' the proportion of red blood cells which were reticulocytes was not significant in the original analyses but was marginally significant for one of the genotypes (AS) in the new analysis. This is keeping with the original analysis on 'resource availability' which found that AS had the strongest response to resource availability.

However, both these effects were only marginal in the original analysis and the main aim of this chapter was to test for genetic variation in patterns of investment, the effect of competition, and the effect of resources when experiencing competition. These findings are clear and robust to the new analysis. The potential impact of autocorrelation for analyses of within-infection dynamics and more detailed statistical methods are presented in chapter 4.

CHAPTER 3

MALARIA AND TRYPANOSOME TRANSMISSION: DIFFERENT PARASITES, SAME RULES?

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3.1. Summary

African trypanosomes produce different specialised stages for within-host replication and between-host transmission and therefore face a resource allocation trade-off between maintaining the current infection (survival) and investment into transmission (reproduction). Evolutionary theory predicts the resolution of this trade-off will significantly affect virulence and infectiousness. The application of life history theory to malaria parasites has provided novel insight into their strategies for survival and reproduction; how this framework can now be applied to trypanosomes is discussed. Specifically, I outline predictions for how parasites trade-off investment in survival and transmission in response to variation in the within-host environment. An evolutionary approach has the power to explain why patterns of investment vary between strains and during infections, giving important insights into parasite biology.

3.2. Protozoan parasites: life history trade-offs

Protozoan parasites, such as African trypanosomes (*Trypanosoma brucei* sp.) and malaria parasites (*Plasmodium* sp.), cause serious mortality and morbidity in humans, livestock and wildlife, and have severe economic impacts in the developing world. These parasites undergo asexual replication within a vertebrate host and must produce specialised transmission stages to be transmitted between hosts by insect vectors. Evolutionary theory predicts that this life cycle results in a trade-off between the investment of resources into survival (replication) and reproduction (production of transmission stages; Box 3.1). Survival versus reproduction trade-offs are a key concept in evolutionary biology and have received a wealth of theoretical and empirical attention (e.g. Fischer *et al.* 2009; McNamara *et al.* 2009; Stearns 1992). Whilst most of the concepts of life history theory have been developed for multi-cellular organisms, single-celled parasites face similar challenges; species competing for resources within a host and being targeted by the immune response are analogous to prey species competing for food and avoiding predators (Graham 2008; Pedersen and Fenton 2007). The predictions of theory are being met with increasing support across a diverse range of taxa, including single-celled parasites (Foster 2005; Griffin *et al.* 2004; Reece *et al.* 2008; Reece *et al.* 2005; West *et al.* 2006).

In recent malaria research, life history theory has provided insight into how parasites respond to selection pressures, such as co-infection with other genotypes or species, or attack from anti-malarial drugs (Mackinnon and Marsh 2010; Mideo 2009; Paul *et al.* 2003; Reece *et al.* 2009). This framework has been successful in explaining the patterns observed in laboratory experiments with model systems (e.g. Buckling *et al.* 1999a; Pollitt *et al.* 2011b; Reece *et al.* 2008; Wargo *et al.* 2007b), and there is also some evidence that these findings are relevant to human malaria parasites in natural infections (Harrington *et al.* 2009) and *in vitro* studies (Buckling *et al.* 1999b; Reece *et al.* 2010). In contrast, trypanosome research has largely remained focussed on molecular and cellular biology (but see Balmer *et al.* 2009). The success of using life history theory to understand the strategies of malaria parasites suggests that this framework can also be applied usefully to trypanosomes to explain variation in parasite strategies, across genotypes and during infections. In this article I discuss

how predictions from life history theory can be applied to understand the investment strategies of trypanosomes. The trade-off between investment in survival (replication) and reproduction (production of transmissible stumpy forms) is focused on for two reasons. Firstly, there are clear and useful parallels with recent findings in malaria parasites (Box 3.2). Secondly, the relative investment in within-host replication and between-host transmission is predicted to have significant effects on virulence and infectiousness (Alizon *et al.* 2009).

Box 3.1. Key concepts in evolutionary ecology

Life history trade-offs

Life history traits are phenotypic components influencing fitness (Stearns 1992). For parasites this includes replication rate and the relative investment into and timing of transmission stage production (Reece *et al.* 2009). While organisms are selected to maximise fitness they are also constrained by trade-offs between different life history traits (Stearns 1992). Trade-offs can take various forms but the most commonly considered result from resource limitation: organisms have limited resources to invest in different life processes and therefore need to balance investment to maximise fitness (Stearns 1992). In metazoans, single organisms are easily identifiable as the target of natural selection. In single-celled parasites, a genotype within an infection is the comparable target (Gardner and Grafen 2009). When infections consist of a single genotype, trade-offs will be resolved across all parasites in the host, maximising overall fitness over the lifetime of the infection (West *et al.* 2006). An important trade-off, especially in long lasting infections such as with malaria and trypanosome parasites, is between current investment in between-host transmission and investment in maintaining the infection (within-host survival) for future transmission (Mideo and Day 2008).

Phenotypic plasticity and fixed strategies

Examining and explaining trade-offs is complicated due to organisms evolving under varying environmental conditions. The best solutions to resource allocation trade-offs depend on the opportunities and constraints offered by the within-host environment and how they change throughout infections (Poulin 2007).

Environmental conditions can lead to changes in life-history traits by two distinct, but not mutually exclusive, processes (Roff 2002). First, organisms may be able to produce a range of phenotypic responses according to variation in environmental or internal conditions. This process, known as adaptive phenotypic plasticity, is central to understanding the effects of environmental variation on evolution and can be broadly defined as a change in the phenotype of a given genotype in response to environmental cues (Pigliucci 2005). This enables organisms to respond rapidly to predictable environmental changes in ways that maximise fitness (Poulin 2007). For example, the fresh water crustacean *Daphnia pulex* produces costly morphological defences, including neck spines, when exposed to predator cues (Hammill *et al.* 2008). Second, with longer-term environmental changes, spanning multiple generations, microevolution can occur where population gene frequencies change due to individuals best adapted to the new conditions disproportionably contributing to future generations (Poulin 2007). For example, when a new high coverage drug treatment is introduced, genes for resistance mechanisms spread in the population of parasites targeted (Palumbi 2001).

3.3. Trypanosomes: survival and reproduction

When an infected tsetse fly bites a mammalian host, metacyclic forms are inoculated into the blood. These develop into slender form parasites that undergo rapid asexual replication, maintaining the infection in the host (survival). As parasite density increases, a parasite-derived factor accumulates (termed 'stumpy induction factor' or 'SIF') and causes some, but importantly not all, parasites to undergo cell cycle arrest and differentiate into stumpy forms (Vassella *et al.* 1997). Stumpy forms have a limited life expectancy in the blood, as they no longer replicate or productively switch their VSG coat, but they are infective to tsetse flies and therefore provide the potential for transmission (reproduction; Roditi and Lehane 2008). Trypanosome infections generally involve cyclical peaks in parasitaemia (Figure 3.1). Statistical modelling indicates that parasite driven differentiation, together with antigenic variation, can generate this pattern, and therefore the distinctive waves of parasitaemia are predominately under parasite control (Lythgoe *et al.* 2007). The role that differentiation plays in generating waves of parasitaemia is supported by observations that laboratory strains which cannot produce stumpy forms continue to replicate quickly killing the host (Turner *et al.* 1995), and that cycles of parasitaemia are still observed in infections of immunocompromised mice (Balber 1972).

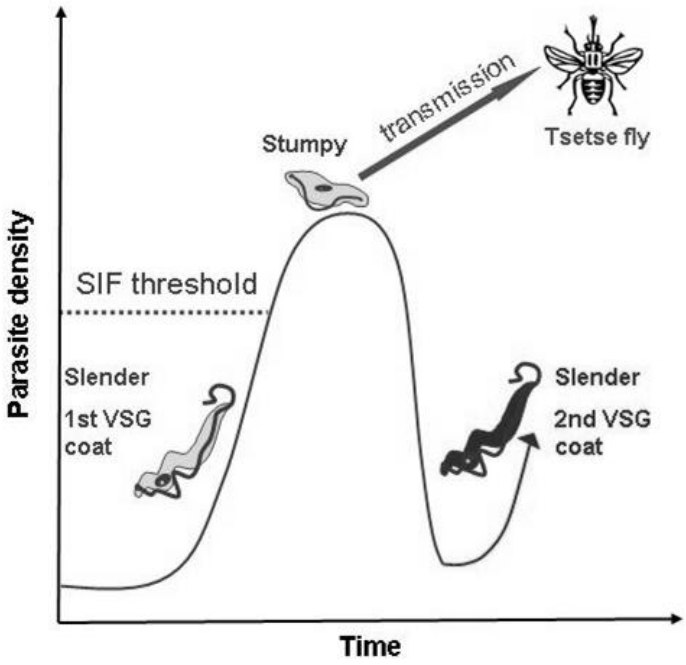

Figure 3.1. Dynamics of trypanosome infection in the mammalian host. As slender form parasites replicate in the blood the parasitaemia rises, as does the concentration of a soluble stumpy induction factor (SIF), inducing some parasites to differentiate into non-replicating, but transmissible, stumpy forms. A combination of differentiation into stumpy forms and clearance, as the immune system mounts a response to the first VSG coat, leads to a crash in parasitaemia. However, as some slender forms have switched VSG coats a second wave of parasites, not yet recognised by the immune system, begins to increase parasitaemia once again.

However, in natural infections, parasitaemia will be shaped by a combination of the host immune response and the production of stumpy forms. To evade the host immune response, trypanosomes employ a strategy of changing their VSG (variant surface glycoprotein) surface coat. Each parasite has a repertoire of thousands of VSG genes but expresses only one at a time (Taylor and Rudenko 2006). Initially in laboratory infections, one or a few VSG variants dominate but the immune system eventually raises antibodies against these coats, leading to wide-scale clearance. Each parasite has a low probability of switching to the expression of a new variant (McCulloch and Horn 2009). Therefore, during every replication cycle, a small number of parasites are likely to have a VSG coat not yet recognised by the immune system, and these parasites will rapidly replicate, resulting in a new wave of parasitaemia (Figure 3.1; McCulloch and Horn 2009). It is important to note that although variants differ in the VSG gene(s) being expressed over the course of a single infection, they are isogenic to the original infecting parasite clone(s). This is significant because natural selection acts at the level of the parasite genotype within infections, therefore clonally related parasites will be selected, as a group, to maximise the transmission of their genotype over the course of the infection (Gardner and Grafen 2009; West *et al.* 2006b).

Each trypanosome faces a choice between differentiation into transmissible a stumpy form and continued division as a slender form. From the perspective of a parasite cohort, continued replication of slender forms is necessary to withstand attack from the immune system; for example, maintaining parasite numbers provides the potential to express new VSG coats, whereas stumpy forms provide the potential for between-host transmission. This trade-off has obvious parallels with gametocyte production in malaria parasites (Box 3.2). Also, like malaria parasites, trypanosomes will experience variation in their within-host environment, both during infections and in different hosts, which is predicted to influence the balance between investment in slender and stumpy forms.

Box 3.2. Malaria parasite investment strategies in response to stress

Malaria parasites replicate asexually within the red blood cells of their vertebrate host but also produce specialised transmission stages (gametocytes). When male and female gametocytes are taken up in a mosquito blood meal they produce gametes and undergo a round of sexual reproduction in order to infect the vector. The trade-off faced by malaria parasites between asexual replication and production of gametocytes is analogous to the growth *versus* reproduction trade-off faced by all sexually reproducing organisms (Reece *et al.* 2009). Getting the right balance is essential; too few transmission stages results in an evolutionary dead end within the host, but too many may lead to the infection being cleared before a transmission opportunity arises.

Mounting evidence from experimental *P. chabaudi* infections in mice and *in vitro* cultures of *P. falciparum* show that malaria parasites vary their investment in gametocytes during infections depending on aspects of their environment (Table 3.1). These patterns initially seem contradictory and confusing but can be explained in a life history framework in which parasites are expected to respond to changes in the constraints and opportunities experienced during infections (Reece *et al.* 2009). Malaria parasites increase investment in gametocytes when experiencing either very good or very poor conditions. When conditions are good (e.g. high density of preferred red blood cells), parasites have plenty of resources and can afford to invest in gametocytes (Pollitt *et al.* 2011b; Reece *et al.* 2005). When conditions are very poor (e.g. high drug doses or severe resource limitation), then continued survival in the host is unlikely, and parasites make a terminal investment in gametocytes to maximise short-term transmission (Buckling *et al.* 1999a; Buckling *et al.* 1999b). More commonly, parasites experience intermediate stress levels (e.g. competition with other strains, low levels of drugs or host immune factors), and are constrained to ensuring within-host survival by reducing investment in gametocytes (reproductive restraint) (Pollitt *et al.* 2011b; Reece *et al.* 2010; Mideo and Day 2008). It is important to note, however, these patterns will be made more complex by the details of interactions between host and parasite genotypes (Wargo *et al.* 2007a).

A life history framework not only explains variation observed in malaria transmission strategies, but these studies have also provided novel insights into other aspects of their biology. The ability to respond to various aspects of their within-host environment reveals parasite mechanisms to detect information about their surroundings. Most strikingly, malaria parasites respond to both the genetic diversity of their infection and the densities of their own genotype and co-infecting con-specifics (Pollitt *et al.* 2011b; Reece *et al.* 2008). This suggests an ability to discriminate between kin and non-kin previously thought limited to complex multicellular organisms.

Table 3.1. Malaria parasite transmission strategies and the within-host environment

Malaria species	Data source	Environmental change	Predicted level of stress	Effect on relative investment in transmission	
P. chabaudi	Experimental infections in mice	Increased resources	Low stress, high quality within-host environment	All six strains studied increased investment in transmission with higher proportions of young red blood cells (reticulocytes) and five of the six and with total red blood cell density.	a.
P. falciparum	Cultures with drug sensitive strains from natural infections with frequent drug treatment	Exposure to low doses of anti-malarial drugs	Intermediate	Decreased investment in transmission for all three susceptible strains studied.	b.
P. chabaudi	Experimental infections in mice	Presence of conspecific competitor	Intermediate	Decreased investment under competition for all three of the strains studied.	a.
P. chabaudi	Experimental infections in mice	Presence of conspecific competitor	Intermediate	Only significant effect was for decreased investment, but this was only observed in one of two host strains and one of two parasite strains	c.
P. chabaudi	Experimental infections in mice	Exposure to erythropoietin, which signals host anaemia	High stress, low quality within-host environment	Increased investment seen in one strain of *P. chabaudi* but not in one strain of *P. vinckei.*	d.
P. chabaudi	Experimental infections in mice	Exposure to high doses of anti-malarial drugs	High stress, low quality within-host environment	Increased investment in both of the two strains studied.	e.
P. falciparum	Cultures of laboratory strains	Exposure to high doses of anti-malarial drugs	High stress, low quality within-host environment	Increased investment seen across all four strains studied.	f.

References: a. Pollitt et al. 2011b b. Reece et al. 2010 c. Wargo et al. 2007a d. Reece et al. 2005 e. Buckling et al. 1999a f. Buckling et al. 1999b

3.4. Strategies to maximise survival and reproduction: evolutionary predictions

Trypanosome parasites reach a threshold before some parasites differentiate into transmissible stumpy forms. Evolutionary theory predicts that, in general, the relative level of investment into reproduction will depend on the quality of the environment and that this relationship will be U-shaped (Fischer *et al.* 2009). For trypanosomes, investment into transmission (differentiation into stumpy forms) should depend on the quality of the within-host environment. Investment in stumpy forms is predicted to be highest under extremely good conditions, when parasites can afford to invest heavily, or extremely poor conditions, where survival is unlikely and parasites employ a strategy of terminal investment. Between these two extremes, parasites will be constrained by investing in within-host survival by adopting reproductive restraint (Figure 3.2a). The strategies observed at the extremes make intuitive sense, but explaining why reproductive restraint is predicted is more complex. When parasites experience stressful (but not terminal) situations, they must produce enough slender replicating forms to maintain the infection, which lowers the density of transmissible stumpy forms in the short term but maximises fitness over the course of the infection (Figure 3.2b). The importance of within-host survival is often overlooked but safe guarding future transmission will be an important determinant of parasite fitness when infections persist over long periods.

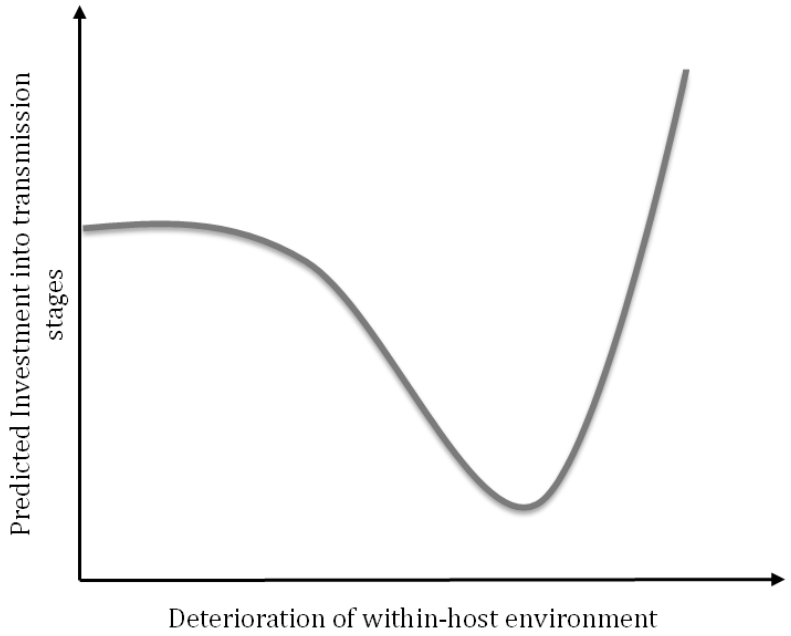

Figure 3.2a Strategies for the relative investment into transmission stages: predicted pattern. Theory predicts that organisms will invest heavily in reproduction under either very good or exceptionally poor conditions, and be constrained to investing in survival in intermediate situations (Fischer *et al.* 2009). When applied to trypanosomes, parasites are predicted to produce high numbers of transmissible stumpy forms in extremely good or extremely poor within-host environments, but, in most conditions be constrained to producing enough slender form parasites to maintain the current infection. As with malaria parasites it is likely that there will be genetic variation between strains for the ability to accurately detect and respond to environmental cues, and the level of stress experienced in a given environment (Pollitt *et al.* 2011b).

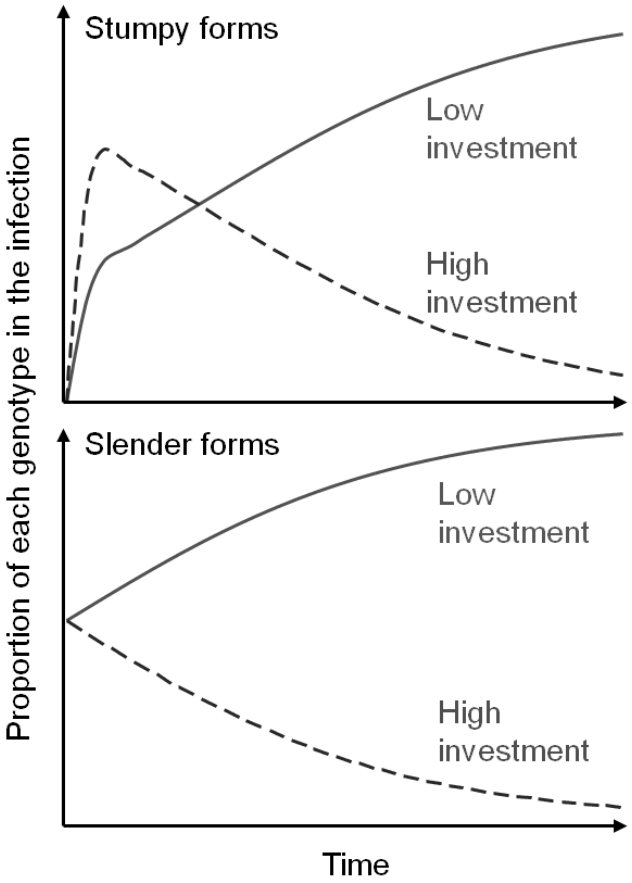

Figure 3.2b Strategies for the relative investment into transmission stages: predicted competitive outcomes. Parasite infections were simulated where a mixed infection was started with two genotypes at equal densities (100 replicating forms of each genotype) but one genotype invested 10% of new cells into producing transmission stages (stumpy forms; solid line – low investment) and one genotype invested 20% of new cells into producing transmission stages (dashed lines – high investment). It was assumed that the two genotypes had the same replication rate and there was no difference in mortality between strains or parasite stages. This simple simulation illustrates how higher investment in transmission stages could give short term benefits (higher chance of being transmitted) but be detrimental to longer term transmission success. The optimal strategy will depend on the chances of surviving in the host and transmission opportunities for the parasite. For example in mixed genotype trypanosome infections then we predict that the low investment strain would have higher fitness as infections can persist for many years.

3.5. Adding ecology

For trypanosomes, like other parasites, key variables determining the quality of the within-host environment include: (i) exposure to immune responses, (ii) availability of the host's resources, (iii) exposure to trypanocidal drugs, and (iv) the presence of competitors. Trypanosomes live freely within the circulation and generate energy through glycolysis of blood glucose. Although the occurrence of hypoglycaemia, at least at peak parasitaemia, is indicative of it being a limiting resource, the effect of glucose level on trypanosome development *in vivo* is yet to be quantified. Similarly, if the efficacy of drugs (where applied) or the force of attack by the immune system varies, trypanosomes will be exposed to different levels of stress. Competitive suppression has been demonstrated to occur in trypanosomes (Balmer *et al.* 2009), and clear parallels can be drawn with the responses of malaria parasites to competitors, which is discussed below. In reality, the overall quality of the within-host environment and the net level of stress parasites experience, is likely to be influenced by interactions between these variables and further complicated by both host and parasite factors. However, as a starting point to develop clear predictions that can be tested with laboratory experiments it is useful to consider these different stresses in terms of where they will place parasites on the axis of environmental quality (Figure 3.2a).

3.6. Within-host competition

Like most organisms, parasites (in genetically mixed infections) encounter competitors, and understanding how this affects parasite traits is receiving attention (Brown *et al.* 2002; Foster 2005; Mideo 2009; Read and Taylor 2001). Trypanosomes in mixed infections are suppressed, resulting in lower parasite densities (Balmer *et al.* 2009). This could be driven by either resource limitation, mixed infections triggering stronger host defences, or direct interference competition between strains (Balmer *et al.* 2009). Increasing investment in replication could ameliorate competitive suppression by enabling parasites to exploit the greater share of host resources and/or the generation of new VSG variants. Evolutionary theory for

malaria parasites predicts that reproductive restraint maximises competitive ability (Mideo and Day 2008) and a recent laboratory study reveals that they employ this strategy when in competition (Pollitt *et al.* 2011b; but see Wargo *et al.* 2007a).

The extent of reproductive restraint parasites should adopt is predicted to depend on the extent of suppression, which is determined by relative competitive ability. For a poor competitor, a mixed infection is likely to be a very bad environment because proliferation is heavily suppressed, and a terminal investment may be the best strategy. In contrast reproductive restraint may be unnecessary for the best competitors who experience the least suppression. These predictions are consistent with observations that malaria parasites with faster replication rates compete more effectively in experimental mixed infections (de Roode *et al.* 2005b) and may be complicated if competitive ability depends on who the competitors are. Furthermore, postponing transmission in the short term to improve competitive ability could be risky if mixed infections are particularly virulent and are likely to rapidly kill the host. However, as natural infections of malaria parasites and trypanosomes are usually chronic and persist over multiple replication cycles and competition suppresses overall parasite density, safe guarding future transmission is likely to be an important component of parasite fitness.

3.7. Complex within-host environments

Importantly, the quality of the within-host environment will be shaped by multiple interacting factors and will vary over the course of infection. For example, parasite interactions between strains are complex, spanning from facilitation to competitive suppression. These interactions will also influence and be influenced by factors including host immunity and resource availability (Brown *et al.* 2008; Mideo 2009; Read and Taylor 2001). Additionally, intrinsic host factors will also be important, for example the rate of SIF turnover or immune competence may vary between individual hosts, leading to complex feedbacks with parasite strategies. The relative importance of different factors, such as competition, immunity and resource limitation, in shaping the quality of the within-host environment, and thus precisely

where they place parasites on Figure 3.2a, is yet to be determined. A combination of using mathematical models to explain experimental data and developing evolutionary theory specifically for trypanosomes will be extremely useful.

3.8. Responding to environmental change

Parasite investment strategies may be fixed, plastic or a combination of both. Whether parasites evolve fixed or plastic responses to cope with changes in the circumstances experienced during infections depends on: the frequency of encountering situations, the benefits of responding, and the costs and the constraints involved (Box 3.1; Poulin 2007; Reece *et al.* 2009). For example, for parasites to plastically alter strategies in mixed infections they must be able to gather information on the genetic diversity of the infection. Bacteria coordinate group behaviours using quorum sensing to transmit and receive information about density and relatedness (Antunes and Ferreira 2009). Malaria parasites also appear to be capable of responding to density and relatedness, although the mechanism is not yet known (Pollitt *et al.* 2011b; Reece *et al.* 2008). Trypanosomes detect and respond to SIF in a density-dependent manner and there is also evidence for the coordination of group motility behaviours in the tsetse infective (procyclic) form (Oberholzer *et al.* 2010).

Given these observations and the extent that the within-host environment varies during infections and between hosts, plastic responses are likely. Trypanosomes could plastically alter investment into stumpy forms by adjusting the amount of SIF produced or their threshold for responding to SIF. Although SIF is yet to be identified (MacGregor and Matthews 2010), experimental work has indicated that it is a small soluble molecule secreted by the replicating slender stages (Vassella *et al.* 1997). Adjusting the concentration of circulating SIF may be complicated by variation in rates of host clearance, and whether SIF initiates a response that is strain-specific or pan-infection. Conditioned media produced by one strain was found to be able to induce stumpy form production in two other strains (Vassella *et al.* 1997), suggesting that SIF may be general across genotypes. Therefore, varying the threshold for responding to SIF may be a better strategy as it could protect parasites

from manipulation by co-infecting strains. Laboratory adapted strains become insensitive to the SIF they produce (Vassella *et al.* 1997); however, it is not yet known if there is a range of sensitivities or whether it is an 'all or nothing' response.

In parasite populations where mixed infections and the resulting competitive suppression are the norm, reduced investment in transmission is likely to become fixed. This could have dramatic effects on virulence to the host: less virulent strains may actually reduce harm by suppressing more virulent strains (Balmer *et al.* 2009). But, as demonstrated for malaria parasites, if these virulent parasites are released from competition (for example, by being the only genotype transmitted, or through selective drug treatment), the brakes could be removed from the replication of the virulent strain and hosts could experience more severe disease (Harrington *et al.* 2009; Schneider *et al.* 2008; Wargo *et al.* 2007b).

3.9. Where do we go from here?

Life-history theory can provide testable predictions for trypanosome investment strategies. However, to move forward it is necessary to perform controlled and rigorous experiments that examine parasite strategies under manipulated (perturbed) within-host conditions. As there are clear predictions for how parasites will respond to competition, and mixed infections are a relatively simple experimental manipulation to perform, within-host competition is a good starting point. The integration of mathematical modelling approaches, with experimental data from these experiments, will be crucial to improve our understanding of the complex interactions within infections and their effect on parasite investment strategies. Mathematical models can tease apart the factors and processes underlying biological patterns to form hypotheses which can be tested empirically (Mideo *et al.* 2008c).

3.9.1. Determining the ecology of mixed infections

There has been little work to quantify the prevalence of mixed infections in trypanosome populations, or their influence on parasite phenotypes. However, field research indicates that there is a range of population structures in African

trypanosomes (MacLeod *et al.* 2001; Morrison *et al.* 2008), as well as genetic variation for traits underlying virulence (Morrison *et al.* 2009). The genetic tools available for *Trypanosoma brucei* (Berriman *et al.* 2005) and large-scale field projects examining the incidence and epidemiology of trypanosome infections could provide a much clearer picture of mixed infections. This requires developing markers to identify, and ideally quantify, different strains. While the ultimate aim will be to understand how the presence of competing genotypes influences trypanosome life history traits and dynamics in natural infections, the first step, as with malaria parasites, will be to perform controlled lab experiments. To do this it will be necessary to increase the number of genetically characterised strains available for experiments. Field strains are available for trypanosomes but are underexploited in experimental settings in favour of laboratory-adapted strains, which although useful for molecular studies, may not provide realistic information on transmission strategies (Matthews *et al.* 2004).

3.9.2. Quantifying investment into transmission stages

The development of genotype and stage specific qRT-PCR for malaria parasites has made it possible to track focal genotypes during experimental infections to quantify their investment decisions (Babiker *et al.* 2008; Wargo *et al.* 2006). For trypanosomes, classification of cells as slender or stumpy has traditionally depended on their morphological characteristics - an unreliable process due to the existence of intermediate forms. However, a gene array named PAD (proteins associated with differentiation) involved in transmission has recently been identified (Dean *et al.* 2009). As PAD marks the transmissible stumpy form, assays to quantify its expression will allow researchers to reliably monitor levels of differentiation over the course of the infection (MacGregor and Matthews 2010). By comparing patterns of investment in transmission stages of focal parasite genotypes, in single and mixed infections, it will be possible to test for plastic responses to competition. Yet, as transmission investment is predicted to be simultaneously influenced by multiple factors (e.g. competition, resource availability, immune responses) as well as variation in their effects on different parasite genotypes, it is important to measure or

control for the effects of potentially confounding variables when examining patterns. To understand dynamics in mixed infections it will also be necessary to examine variation in the response to SIF produced by clone mates and other strains across a range of genotypes from areas where mixed infections are common. Again, controlled experiments will be the first step before analysis of samples from natural infections.

3.12. Conclusions

Evolutionary ecology can explain parasite traits and uncover strategic (adaptive) patterns in what often seems to be noisy data (Poulin 2007; West *et al.* 2006). Trypanosomes provide exciting opportunities for integrating evolutionary biology with parasitology. As much of their molecular biology is well understood, and there are highly tractable tools for reverse genetic analysis, the mechanisms underpinning parasite traits, such as kin discrimination, may be relatively straightforward to identify. In this way, research into trypanosome life-history strategies can feed back into malaria research where these mechanisms are not yet understood. By explaining parasite life-history traits it will be possible to gain insight into how, when, and why traits underlying transmission and virulence vary, which will lead to better informed control strategies.

CHAPTER 4

TEMPORAL AUTOCORRELATION AND MIXED EFFECT MODELS IN PARASITOLOGY

4.1. Summary

The complexity of the within-host environment poses specific statistical challenges for examining parasite life history traits, and within infection variation means that single measurements from hosts may overlook important interactions or give misleading results. The increased use of mixed effect models allows researchers to use repeated measures designs to examine temporal dynamics of complex and variable within-host environments. However, to ensure the robustness of findings, the inherent assumptions of these models must be understood and satisfied. Here, I use simulated and real infection data to show how temporal autocorrelation of model residuals can lead to type 1 errors (false positives). I then show how this can be prevented by fitting appropriate error structures into models, allowing for the reliable analysis of repeated-measures infection data.

4.2. Introduction

Evolutionary ecology aims to explain the genetic and phenotypic variation observed as a result of ecological processes and interactions between organisms (Stearns 1992; Stearns and Hoekstra 2000). Applying theory from evolutionary biology and ecology to the study of infectious disease has improved understanding of how the parasite phenotypes underlying disease transmission and pathogenesis are shaped by within-host ecology (Brown *et al.* 2002; Combes 2001; Day 2003; Foster 2005; Frank 2002; Galvani 2003; Paul *et al.* 2003; Stearns and Koella 2008; Williams and Nesse 1991). In addition to theoretical frameworks, evolutionary ecology can inform parasitological research through the application of advanced statistical methods. For example, mixed effects models are commonly used in studies of behaviour, life-history and evolution of wild mammals in variable environments (e.g. Bell *et al.* 2009; Reed *et al.* 2008; Stopher *et al.* 2011) and these type of analyses can also provide powerful tools to assist in understanding the effect of multiple interacting factors on infection dynamics (Paterson and Lello 2003). However, while the advent of more sophisticated analysis is generally a positive advance; such increased sophistication often comes at the price of more complex assumptions.

A common problem in parasitological data is pseudo-replication (Hurlbert 1984), when repeated measurements are taken from the same individual, or groups of individuals experiencing the same conditions (e.g. a cage of mosquitoes or multiple time-points across an infection; Paterson and Lello 2003). These measurements are not independent measurements of treatment effects and, if treated as such, will violate assumptions and potentially lead to spurious results – type 1 errors. For example, in laboratory studies of malaria infection, some mice may respond more strongly to a treatment and consistently have higher, or lower, trait values, or be influenced by factors outside the experimental design. One solution is to avoid pseudo-replication through experimental design. For example, it may be possible to design the study in such a way that each measurement is independent, i.e. sampling different individuals for each time point. This will remove the problem of pseudo-replication and in some cases (e.g. where destructive sampling is necessary) is the obvious choice. However, in many cases there will be ethical, financial and time

considerations which make this approach unfeasible. The problem of pseudo-replication could also be eliminated by using summary statistics to represent the infection or group of individuals with one value e.g. peak parasitemia, maximum anaemia or cumulative gametocyte production. This approach ensures that values are independent and therefore allows for the use of standard statistical methods (linear models, t-tests, etc). However, infections are complex and dynamic; multiple interacting factors shape parasite traits, and within-host environments vary over time and between different niches. Therefore, studies examining infections as a snapshot in time are likely to miss a large degree of complexity and subtle (and relevant) variation in patterns, and consequently risk obtaining misleading results or missing confounding effects (Färnert 2008).

An alternative approach is to control for pseudo-replication in statistical analyses. This can be done by including a term in the model (e.g. mosquito cage or mouse identity) to describe the innate variation in each individual/group before testing for the effect of treatment or other variables of interest (Quinn and Keough 2002). With traditional techniques such as ANOVA, this approach used up a degree of freedom for each individual/group and also generally required balanced and orthogonal data (i.e. all treatment combinations must be present and have the same number of observations). However, the advent of mixed effect modelling has relaxed this requirement, allowing for single parameters to describe consistent variation between groups or individuals, and has therefore made the analysis of repeated measure data more attractive (Zuur *et al.* 2007; Zuur *et al.* 2009).

Mixed effect models work by fitting fixed effects, random effects, and error terms into the model. Fixed effect terms explain the variation due to the treatment or variable or interest (e.g. drug vs. no drug, competitor vs. no competitor). Random effect terms, which are specific to a particular group of observations, describe the constant deviation from the mean of some individuals or groups of individuals. For example, in an experiment where measurements of parasite density are taken from individuals over time, some individuals will be inherently more susceptible than others and a random effect term describes the variance that is due to these innate differences (rather than the treatment/environment effects of interest). Finally, error

terms describe the variation (the residuals) remaining in the data that is not explained by either the fixed or the random terms (Pinheiro and Bates 2000; Zuur *et al.* 2009). The use of mixed effect models allows researchers to maximise the degrees of freedom available to detect biological effects and can help 'clean up' noisy data by accounting for the variation due to individuals or groups, meaning that the effect of fixed factors of interest can be seen more clearly (Paterson and Lello 2003). Furthermore, because this approach allows the variation between individuals or groups to be modelled explicitly, the relative contribution of different factors to this variation can be tested (Bolker *et al.* 2009; Elston *et al.* 2001).

However, while fitting a random factor into a mixed effect model accounts for innate effects of individuals which are constant across the whole data set, there is an additional issue relating to the independence of the residuals (the error) which should be considered. The independence assumption states that the residuals should not be correlated in time or space (Zuur *et al.* 2007; Zuur *et al.* 2009). For infection data this assumption will often not be realistic. For example, imagine a hypothetical study which examines the impact of a treatment regime on parasite density over the course of an infection. Replicate infections are initiated in mice and measurements of parasite density are taken each day, from each mouse, over the course of the infection. Some mice will be more susceptible than others, and consistently have a higher density of parasites than the mean for their group. A random effect of mouse is fitted in order to account for this consistent deviation. However, an additional problem is that for each mouse there may still be correlation in the error term between different days. Imagine that some unrecorded factor triggers a strong immune response on day 7 and leads to values being lower than expected on that day. This effect is likely to still be present on day 8 and, consequently, the residuals for day 7 and 8 are not independent. This effect is referred to as 'temporal autocorrelation in residuals' and if not controlled for, can distort statistical inferences by incorrectly inflating test statistics and increasing the likelihood of type 1 errors (Schabenberger and Pierce 2002).

Whilst this issue has been known and discussed across various fields in biology, including ecology, psychology, and neuroscience (e.g. Sarter and Fritschy 2008;

Schabenberger and Pierce 2002; Zuur *et al.* 2009), it is generally not appreciated in parasitology. In a review outlining the utility of mixed effect models for parasitology, Paterson and Lello (2003) mention the autocorrelation assumption. However, a literature search of papers published in *The International Journal for Parasitology* in the 8 years since this review, found that out of 17 papers using mixed effect models to analyse data sets with repeated measurements, only 6 reported checks for autocorrelation. This journal, that publishes original research, has the highest impact factor in parasitology, and has a history of providing statistical guidance (e.g. Morrison 2002), suggesting that analyses in other journals may be less statistically robust. While temporal autocorrelation could be a substantial issue for the field of parasitology very little is known about how common it is in infection data. Therefore, the implications for statistical inference and the understanding of disease are unclear.

Here, I use simulated infection data sets to examine the potential impact of autocorrelation on type 1 error rates. I then investigate the levels of autocorrelation in several real data sets from experimental malaria infections in mice. Finally, I show which methods can be used to reliably analyse repeated-measures data with autocorrelated residuals.

4.3. Methods

4.3.1. Simulating data sets with known autocorrelation

I used R version 2.12.1 (The R foundation for statistical computing; http://www.R-project.org) to simulate artificial data sets with a known amount of autocorrelation in the error structure. Artificial data sets were designed to be simplified versions of those commonly generated in malaria life-history studies, like those presented in chapter 2. Data sets consisted of two treatment groups (n = 10 per group) with measurements on 16 consecutive days. In the simplest case values were generated using:

$$y_{i,t} = Tr_t + \varepsilon_t + R_i,$$

where $y_{i,t}$ is the observation (response variable) for individual (i) at time t and is calculated from the treatment group mean (Tr) and the error (ε) at time t plus the individual intercept (R_i). Tr is held constant across all individuals, treatments, and time points; R is randomly generated from a normal distribution, with a mean of 0 and a standard deviation of 1, and is held constant across time points; and ε is randomly generated from a normal distribution with a mean of 0 and a standard deviation of 1 for each time point. In order to investigate the effect of temporal autocorrelation I introduced differing amounts of 'carry over' (e.g. error which is generated on one day and still present on the next) in the residuals of the data set using:

$$y_{i,t} = Tr_t + \rho(\varepsilon_{t-1}) + 1\text{-}\rho(\varepsilon_t) + R_i,$$

where ρ describes the level of temporal autocorrelation. Formally, ρ is the proportion of the error in the current day's measurement that is attributed to error 'carried over'

from the previous day (i.e., the $\rho(\varepsilon_{t-1})$ term), while a proportion 1-ρ of the error is newly generated (i.e., 1-$\rho(\varepsilon_t)$).

To establish the effect of violating the autocorrelation assumption in the data, I built linear mixed effect models with the individual effect fitted as a random effect term, and fitted treatment, day and the interaction between treatment and day as fixed factor terms. 10,000 individual replicate data sets were generated and the proportion of statistically significant results ($p<0.05$) was recorded for each of the terms in the model. To confirm that the generated data sets resulted in the expected levels and structure of temporal autocorrelation, I both visually examined the autocorrelation structure and recorded autocorrelation estimates. To visually examine the autocorrelation structure of residuals in the models, I used the ACF function in R. This function plots the correlation between residual values from time points across different temporal lags (Crawley 2007). To put a quantitative value on this autocorrelation in the residuals of the models, I used the autoregressive model (AR1) for autocorrelation function. This function is the simplest and one of the most useful autoregressive models, describing the residual (ε) at time t as a function of the residual at time t-1 along with noise (η), which is allowed to vary over time (Pinheiro and Bates 2000).

$$\varepsilon_t = \rho\varepsilon_{t-1} + \eta_t$$

The parameter ρ is the unknown measure of autocorrelation and is estimated from the data. If ρ=0.5, the correlation between residuals separated by one unit of time is 0.5 and the correlation between residuals separated by 2 units of time is 0.5^2 or 0.25 (Zuur *et al.* 2009).

4.3.2. Estimating autocorrelation from real data sets

To investigate the level and structure of autocorrelation in real infection data, I used data from studies of the temporal dynamics of malaria parasite infections.

Data set 1: P. yoelii: The first data set came from a large scale study designed to test the effect of the within-host environment on the infection dynamics of *P. yoelii* malaria parasites in male MF1 mice (Ramiro, Pollitt, Mideo and Reece unpublished data). The data set consisted of measurements from 39 mice divided between two cohorts and three treatments (Control, anaemic and immune). The first cohort was sampled on even days and the second cohort was sampled on odd days between day 4 and 23 post infection. Each infection therefore contributed data to 10 time points. Data was available on five different infection characteristics: mouse weight, red blood cell density (RBC), reticulocyte density (Retics), and densities of malaria parasites infecting either mature (iRBC) or immature red blood cells (iRetics).

Data set 2: P. chabaudi: The second data set came from experiments designed to examine genetic variation for investment into gametocytes and sex ratios in experimental infections of *P. chabaudi* malaria parasites in male MF1 mice (Pollitt *et al.* 2011b; Reece *et al.* 2008). Measurements were taken on each day over the acute period of the infection (day 5 to 12 post infection) for six wild type *P. chabaudi* genotypes (AJ, AS, ER, CR, CW and DK), in 5 replicate infections per genotype. Data was available on seven different infection characteristics: mouse weight, RBC, asexual density, retics, investment into gametocytes (conversion rate; see Buckling *et al.* 1997), and gametocyte sex ratio (S.R.; see Reece *et al.* 2008).

In both cases, the experimental design and sampling regime generating this data was similar to many published studies (e.g. Barclay *et al.* 2008; Bell *et al.* 2006; Huijben *et al.* 2010; Long *et al.* 2005; O'Donnell *et al.* 2011; Reece *et al.* 2008). For both data sets, linear mixed effect models were built using mouse as a random factor to account for the pseudo-replication of repeated measurements. For data set 1, the two cohorts were analysed separately with each of the five infection characteristics as a response variable against day, treatment group and the interaction between treatment group and day. For data set 2, each of the seven infection characteristics was fitted as

a response variable against day. For both data sets, day was fitted as a fixed effect factor to account for the non-linearity of temporal relationships. As with the simulated data set, I used the ACF function in R and the autoregressive model (AR1) to estimate the structure and level of autocorrelation in these real data sets. A visual comparison of the error structures in the simulated data (representative examples shown in figure 5.1a) and the real data sets (representative examples shown in figure 4.1b) showed an autoregressive structure, i.e. there is significant correlation of residuals for data points next to each other in time (lag of 1) and this decreases with the time lag between points.

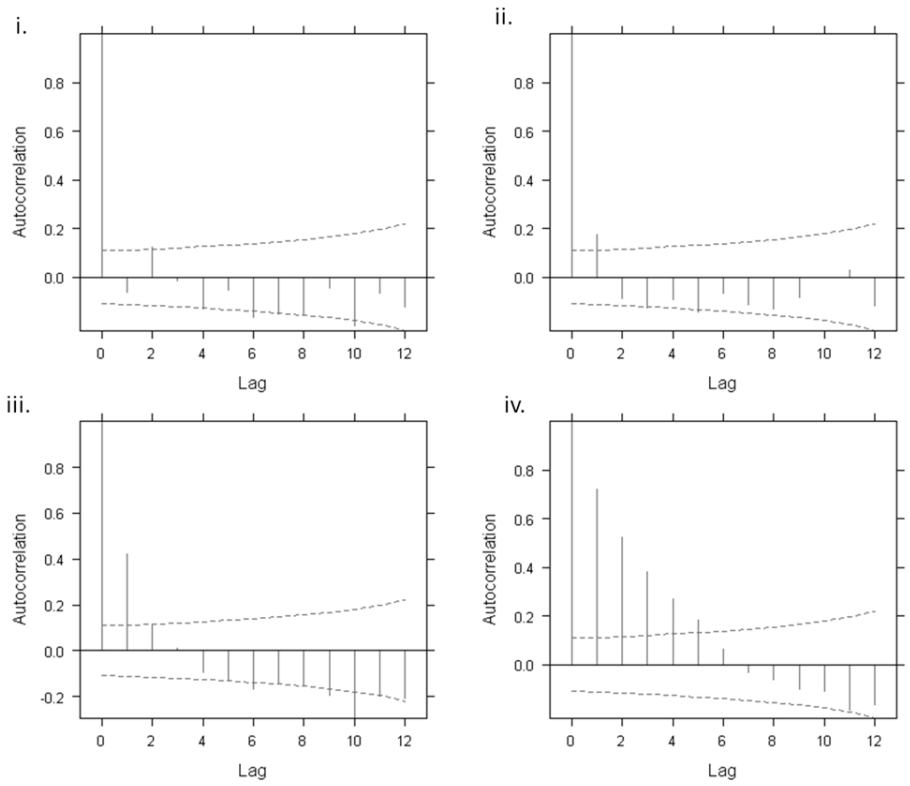

Figure 4.1a. Example autocorrelation structures from simulated data sets with varying levels of autocorrelation in residuals. i. no autocorrelation. ii. 0.25 autocorrelation. iii. 0.5 autocorrelation. iv. 0.75 autocorrelation. Lag refers to the time lag between data points (e.g. lag of 1 = adjacent days) and the dashed lines show the significance cut off with alpha set at 0.05. Autocorrelation at a time lag of zero is 1 as this correlates points against themselves. Structures slightly vary between runs but the above panel shows representative examples.

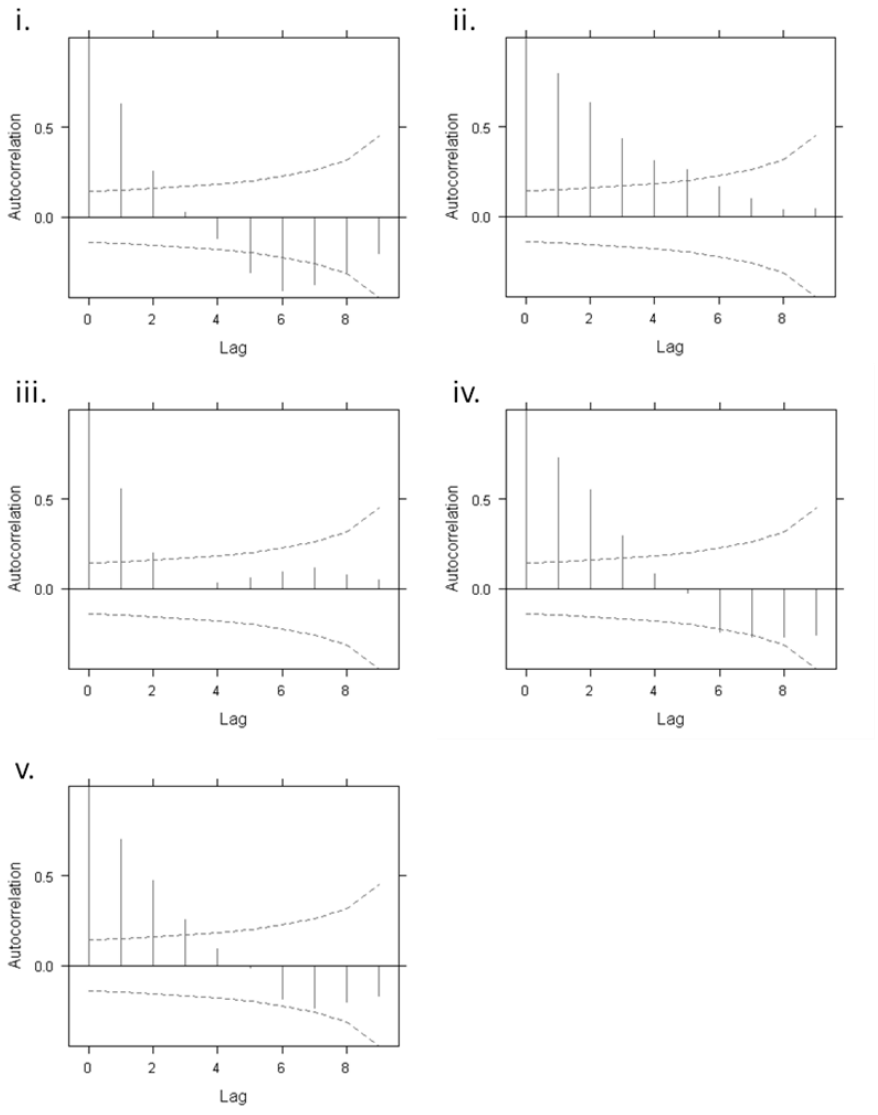

Figure 4.1b. Autocorrelation structures from analysis of real infection data. Example autocorrelation structures presented for *P. yoelii* infections in mice (data set 1, cohort 2). Models examine the effect of day*treatment on: Mouse weight (i), RBC count (ii), Reticulocyte density (iii), Density of infected cells (iv) and density of infected retics (v). Lag refers to the time lag between data points. As mice were sampled on alternate days, lag of 1 = 2 days, lag 2 = 4 days etc. The dashed lines show the significance cut off with alpha set at 0.05.

4.3.3 Correcting for autocorrelation

There are various ways in which the violations of the autocorrelation assumption can be dealt with. In neuroscience, corrections which adjust the p value to make tests more conservative are commonly used (Sarter and Fritschy 2008). However, this approach is not straightforward, as there are multiple ways of calculating the correction (e.g. Greenhouse-Geisser or Huynh-Feldt) and some of these are over-conservative, risking type 2 errors (Quinn and Keough 2002). An alternative approach is to fit error structures into models to account for the autocorrelation. There are various possible error structures which can be used. However, for repeated measures designs, one that might be intuitively be expected to work (and appears to fit models well) assumes that the correlation decreases with lag time, i.e. measurements on day 4 of an infection will be more similar to day 5 than to day 20 (Schabenberger and Pierce 2002). This type of error structure matches the autocorrelation observed in the simulated and real data presented here (figures 4.1a & b), and can be modelled - and therefore controlled for - using autoregressive error structures (Pinheiro and Bates 2000).

In R correcting for autocorrelation of the type shown here is done using the corAR1 correlation function (Zuur *et al.* 2007; Zuur *et al.* 2009). This function is coded by specifying a time covariate (i.e., the unit over which measurements are autocorrelated - in this case, day post infection) and, optionally, a grouping factor (i.e., the level at which the autocorrelation occurs - in this case, mouse). Initially, I fitted corAR1 to the simulated data sets and estimated the type 1 error rates with and without the error structure. I also performed model comparisons using a likelihood ratio test to estimate the proportion of times that corAR1 would significantly improve the fit of the model to the data (Crawley 2007). I then ran analyses for both real data sets with and without the autocorrelation error structure and compared the results of these analyses.

4.4. Results

4.4.1. The impact of autocorrelation on type 1 error rates

As I built temporal autocorrelation into the simulated data sets, I predicted that this would influence the chance of falsely detecting significant effects of day and the interaction between treatment and day. Detecting whether a particular treatment influences parasite dynamics over time is often the aim of infection studies, so I specifically examined the impact of autocorrelation on the interaction term.

A P value gives the probability of obtaining a test statistic equal to or larger than the one observed if the null hypothesis is correct. This null hypothesis implicitly includes the assumptions of the test and therefore if the assumptions are violated, the reported P value will be incorrect. With hypothesis testing the decision whether to accept or reject the null hypothesis is based on a critical P value threshold, which then gives us our accepted type 1 error rate. Since in my simulated data sets the null hypothesis is true - any relationship between treatment group and time is due to chance - if the test is performing correctly it should, by definition, return a P value of ≤ 0.05, 5% of the time. This was the case for the data set with no autocorrelation of error terms (proportion of significant results 0.05 ($\pm 4.3 \times 10^{-3}$ 95% CI)). However, as the level of autocorrelation increased up to 0.75, the proportion of times a P value of ≤ 0.05 was returned rose to 0.48 (± 0.02 95% CI) meaning a type 1 error occurred almost 50% of the time (Figure 4.2).

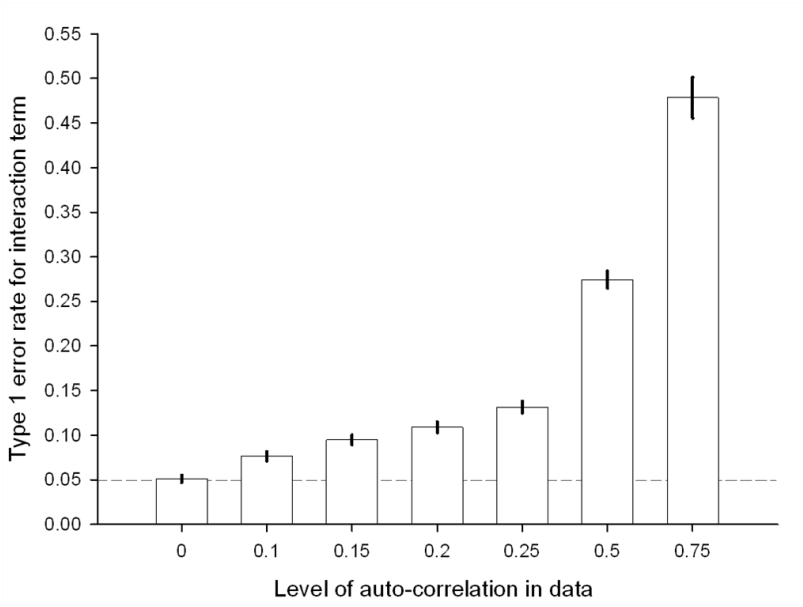

Figure 4.2: Proportion of false statistically significant effects detected with varying degrees of autocorrelation in simulated data sets. Bars show the type 1 error rate (proportion of analyses where $p<0.05$) for the interaction between day and treatment. The dashed reference line shows a 5% type 1 error rate which is the level assumed for mixed effect models and is used as a basis to reject null hypotheses. Means are based on 10,000 runs from simulated data sets and the error bars show 95% confidence intervals.

4.4.2. Levels of autocorrelation in malaria infection data

For data set 1 (*P.yoelii*), the level of autocorrelation varied depending the variable examined; values ranged from 0.49 to 0.96 and varied between the two experimental cohorts (Figure 4.3a). For data set 2 (*P. chabaudi*), values ranged between 0.037 and 0.87, and varied depending on the variable examined and the focal genotype (Figure 4.3b).

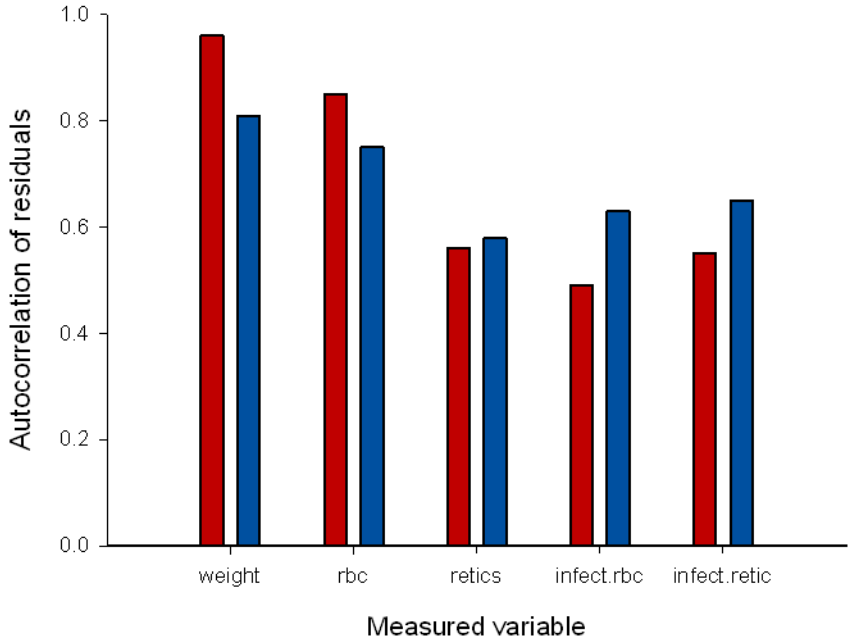

Figure 4.3a: Levels of autocorrelation in infection data sets: Data set 1. Autocorrelation of residuals in models of 5 infection variables measured for *P. yoelii* across cohort 1 (red) and cohort 2 (blue).

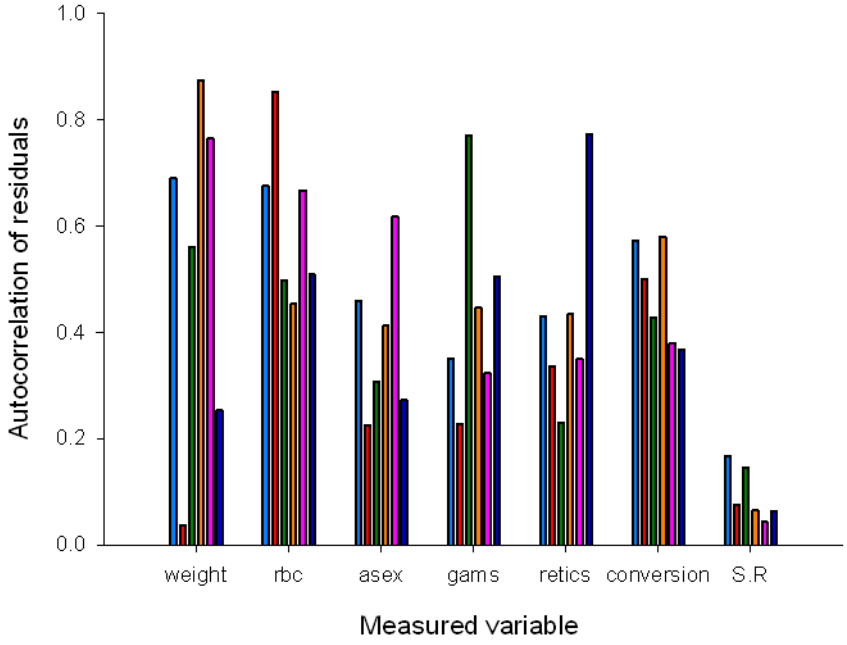

Figure 4.3b: Levels of autocorrelation in infection data sets: Data set 2. Autocorrelation of residuals in models of 7 measured variables for 6 wild type genotypes of *P. chabaudi* (Pale blue = AS, Red = AJ, Green = ER, Orange = DK, Pink = CW, Dark blue = CR).

4.4.3. Fitting corAR1 error structure

Fitting the corAR1 autocorrelation structure to models of the simulated data sets reduced the type 1 error rate (Figure 4.4a). With high levels of autocorrelation, the type 1 error rate was still slightly higher than the generally accepted threshold of 0.05, but still far lower than without corR1. For example, 75% autocorrelation resulted in a type 1 error rate of 0.089 (±0.01 95% CI) with corAR1 and 0.48 (±0.02 95% CI) without corAR1. Comparing model fits showed a steady increase in the proportion of models fitting the data significantly better with corAR1 than without, until 0.25 autocorrelation was reached at which point 100% of model fits were improved by corAR1 (Figure 4.4b).

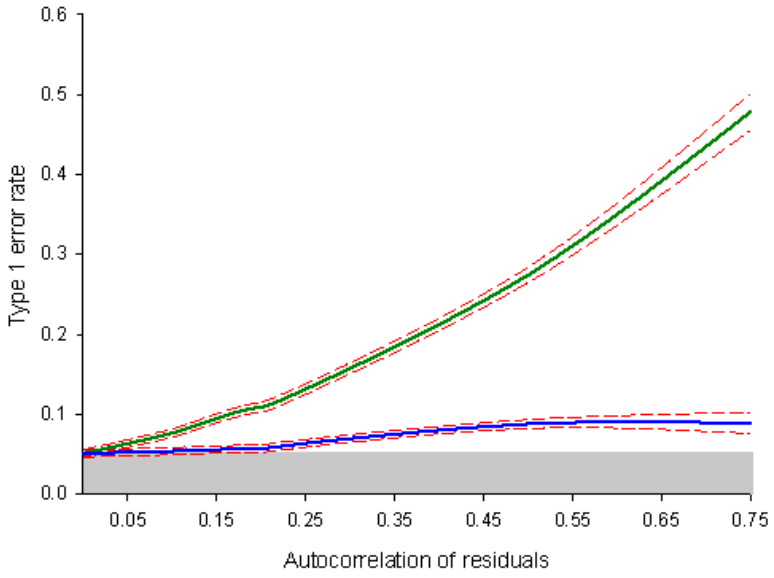

Figure 4.4a: The effect of fitting corAR1 to simulated data sets: type 1 error rates. Type 1 error rates for models, with (blue), or without (green), the corAR1 error structure. The shaded area provides a reference for an error rate of 0.05. Lines are based on a minimum of 10,000 runs and red dashed lines show the 95% confidence intervals.

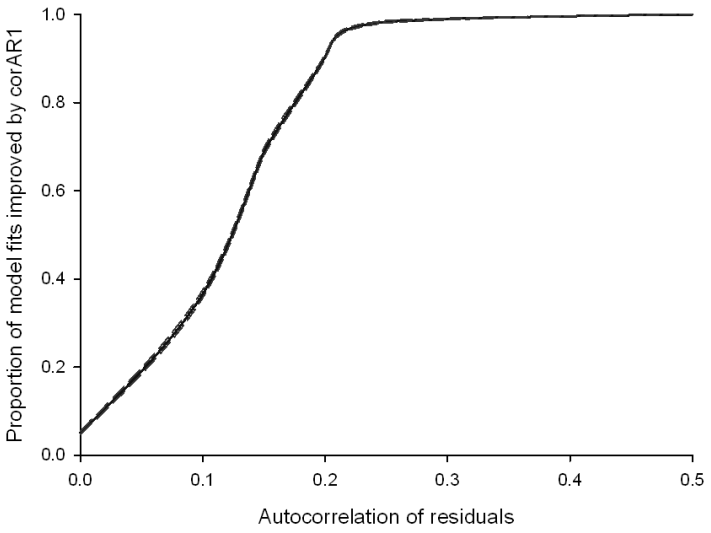

Figure 4.4b: The effect of fitting corAR1 to simulated data sets: model comparison. The proportion of times a model with corAR1 significantly improves the fit of the model to data. Lines are based on a minimum of 10,000 runs and dashed lines show the 95% confidence intervals.

I then used data set 1 to compare analyses performed with and without corAR1. For all response variables in both cohorts, the model including the corAR1 error structure was a significantly better fit for the data than the model without this error structure (table 4.1). Fitting the error structure altered whether the interaction between day and treatment would be judged to be significant (at the 95% confidence level) for 3 out of 5 infection variables in cohort 2, but none in cohort 1 (table 4.1). For the three variables where statistical inference was altered, fitting the error structure revealed a significant effect of two variables (decreasing the p value), and made the significant effect of the third variable marginal (increasing the p value). These results clearly demonstrate that not controlling for temporal autocorrelation can lead to both type 1 and type 2 errors. Data set 2 was also analysed with and without the corAR1 error structure, in order to verify findings in Pollitt *et al.* (2011b). The results of this are reported in appendix 4.6, but broadly support the original analyses.

Table 4.1: Effect of fitting the corAR1 to analyses of data set 1. Results from the interaction term (day*treatment) of linear mixed effect models, with or without a corAR1 error structure fitted. Results highlighted in bold indicate analyses where fitting an autocorrelation structure alters statistical inference (at the 95% confidence level). ρ indicates the estimated level of autocorrelation in residuals.

Cohort 1				
Trait~ day*treat	**ρ**	**Without corAR1**	**With corAR1**	**Model comparison**
Mouse weight	0.96	F=2.52, p=0013	F=1.73, p=0.04	χ^2_1=187.99,p<0.0001
RBC count	0.85	F=6.50, p<0.0001	F=3.48,p<0.0001	χ^2_1=134.53,p<0.0001
Retic density	0.56	F=1.37, p=0.15	F=0.86,p=0.63	χ^2_1=47.47, p<0.0001
Infected cells	0.49	F=2.10, p=0.0083	F=1.95,p=0.016	χ^2_1=37.91, p<0.0001
Infected retics	0.55	F=3.67, p<0.0001	F=2.57, p=0.001	χ^2_1=50.44, p<0.0001
Cohort 2				
Trait ~ day*treat	**ρ**	**Without corAR1**	**With corAR1**	**Model comparison**
Mouse weight	0.81	F=11.19,p<0.0001	F=3.86,p<0.0001	χ^2_1=95.74, p<0.0001
RBC count	0.75	F=3.81, p<0.0001	F=3.17,p<0.0005	χ^2_1=85.54, p<0.0001
Retic density	**0.58**	**F=2.36, p=0.003**	**F=1.66, p=0.054**	**χ^2_1=52.17,p<0.0001**
Infected cells	**0.63**	**F=1.35, p=0.16**	**F=2.04, p=0.011**	**χ^2_1=74.21,p<0.0001**
Infected retics	**0.65**	**F=1.71, p=0.44**	**F=2.40, p=0.002**	**χ^2_1=85.47,p<0.0001**

4.5. Discussion

Analysing the data from experimental infections of two species of malaria parasites indicates that levels of temporal autocorrelation in infection data sets can be extremely high and vary markedly depending on: genotypes, the response variable examined, and even between cohorts from the same study. Some response variables seem to be less affected (e.g. sex ratio; figure 4.3b), however variation in the strength of autocorrelation does not follow any clear pattern according to experimental design, parasite species or genotype. While this makes predicting the effect of temporal autocorrelation for experimental design and analysis more challenging, understanding the reasons for this variation may give biologically interesting insight on processes occurring within infections.

The analyses of both simulated and real data sets demonstrate the substantial effect of violating the assumption for independence of residuals on type 1 error rates. Therefore, the failure to test or account for temporal autocorrelation could substantially alter statistical inferences and, in the worst case, lead to the publication of falsely positive results. Additionally, my analysis of real data demonstrates that by fitting an autocorrelation error structure into models and improving the fit of the model, it may be possible to detect effects which would otherwise be missed, therefore also avoiding type 2 errors. While the analysis presented here focuses on malaria data, I expect that if such high levels of autocorrelation are observed in these data sets from different parasite species and spanning a wide range of response variables, it is likely to be a general issue across infection data.

I have shown that functions are available in R to deal with temporal autocorrelation, and can successfully be fitted in models to reduce type 1 error rates to near expected levels. The corAR1 error structure assumes that time points next to each other will be the most highly correlated and that this will decrease as the time lag between points increases. There are other, more complex, correlation structure functions available (see Zuur *et al.* 2009) and the best structure to use should be judged from visual examination of the correlations between residuals from different time points (e.g. figures 4.1a & b). There may be cases where other structures produce a better model fit. For example, if the degree of temporal autocorrelation is much higher in late

rather than early infections then a more complex moving average structure (ARMA) which estimates the autocorrelation across different points rather than estimating the overall level may be preferable (see Pinheiro and Bates 2000; Zuur *et al.* 2009). However, more complex structures will use up more degrees of freedom and, as with all modelling, there is a trade-off between complexity and the ability to make any real biological inferences. Further, matching the error structure perfectly is not necessary as the differences in the p values obtained by using similar correlation structures will often vary only very marginally (Schabenberger and Pierce 2002; Zuur *et al.* 2009). Therefore, the AR1 error structure presented here is likely to be appropriate for the majority of time course data (Schabenberger and Pierce 2002).

The easiest way to test for temporal autocorrelation is to fit models with and without the error structure and compare the model fit using a likelihood ratio test. This will reveal if AR1 has significantly improved the fit of the model to the data, and which model should be used for making statistical inferences (e.g., if AR1 significantly improves the fit of the model to the data, then statistical inferences should be drawn from the model including this term). There are however limitations of the corAR1 function as some models will not allow for an autocorrelation error structure to be fitted. For example, in the analysis of competition effects presented in chapter 2 (Pollitt *et al.* 2011b), multiple focal genotypes were tracked during infections. This maximises power while minimising the number of mice used. However, it is not possible to fit an AR1 error structure when individuals contribute multiple observations to one time point, and, in this case, analysis needed to be split to examine each genotype independently. Therefore, there may be a trade-off between maximising power by analysing all data together and ensuring the robust nature of results by splitting data to allow for AR1 to be fitted. However, the analyses presented here suggest that temporal autocorrelation should always be considered.

In conclusion, I would strongly encourage parasitologists to test for autocorrelation when analysing temporal patterns in data. Fitting mixed effect models with and without an AR1 autocorrelation structure and comparing the fit of both models to the data is a relatively straightforward procedure in R and may prevent incorrect statistical inferences. Furthermore, future work examining how levels of

autocorrelation vary across a greater range of settings may provide interesting insights into processes driving within-host dynamics.

CHAPTER 5

INVESTIGATING THE EVOLUTION OF APOPTOSIS IN MALARIA PARASITES: THE IMPORTANCE OF ECOLOGY

This chapter is published in Parasites and Vectors as: Pollitt, L. C., N. Colegrave, S.H. Khan, M. Sajid and S. E. Reece. 2010. Investigating the Evolution of Apoptosis in Malaria Parasites: the Importance of Ecology. 3: 105

5.1. Summary

Apoptosis is a precisely regulated process of cell death which occurs widely in multicellular organisms and is essential for normal development and immune defences. In recent years, interest has grown in the occurrence of apoptosis in unicellular organisms. In particular, as apoptosis has been reported in a wide range of pathogen species, including protozoan malaria parasites and trypanosomes, and may provide a novel target for intervention. However, it is important to understand when and why parasites employ an apoptosis strategy before the likely long- and short-term success of such an intervention can be evaluated. The occurrence of apoptosis in unicellular parasites provides a challenge for evolutionary theory to explain as organisms are expected to have evolved to maximise their own proliferation, not death. One possible explanation is that protozoan parasites undergo apoptosis in order to gain a group benefit from controlling their density to prevent premature vector mortality. However, experimental manipulations to examine the ultimate causes behind apoptosis in parasites are lacking. In this review, I focus on malaria parasites to outline how an evolutionary framework can help make predictions about the ecological circumstances under which apoptosis could evolve. I then highlight the ecological considerations that should be taken into account when designing evolutionary experiments involving markers of cell death, and call for collaboration between researchers in different fields to identify and develop appropriate markers in reference to parasite ecology and to resolve debates on terminology.

5.2. Introduction

Apoptosis is a controlled process of programmed cell death (PCD) by which unwanted or damaged cells are eliminated (Popov and Korochkin 2004; Saran 2000). In metazoans, the apoptosis pathway was first described over 35 years ago (Kerr *et al.* 1972), and is now recognised as essential for normal growth and development, as well as helping to guard against infections and the onset of cancer (Popov and Korochkin 2004; Meier *et al.* 2000). The process of apoptosis is initiated by the activation of death receptors, or by intracellular stress conditions (Sayan *et al.* 2001). This leads to a series of genetically controlled and ordered biochemical changes, resulting in morphological changes to the cell (Sayan *et al.* 2001). These include the condensing of chromatin, DNA breakdown, membrane changes, shrinkage of the cell and finally the formation of apoptotic bodies (Kroemer *et al.* 2009). The membrane changes involved in apoptosis act as a signal for apoptotic bodies to be taken up by macrophages, preventing inflammation, as well as passing on information to scavenger cells on the cause of death (Savil and Fadok 2000). In mammals, the process of apoptosis is rapid, removing cells within hours of initiation without evoking the inflammatory arm of the immune system (Savil and Fadok 2000).

Traditionally, apoptosis was thought of as a cellular activity exclusively relevant to multi-cellular organisms, but this view has recently been challenged. Morphological changes during cell death that are consistent with PCD have been reported for a range of unicellular organisms, including protozoan parasites (Ameisen 2002; Bruchhaus *et al.* 2007; Debrabant *et al.* 2003; Deponte 2008; Gordeeva *et al.* 2004). The number of studies revealing PCD markers in unicellular organisms is rapidly increasing, and range across bacteria (Sat *et al.* 2001), slime moulds (Cornillon *et al.* 1994), yeast (Fröhlich and Madeo 2000; Madeo *et al.* 2002a), algae (Moharikar *et al.* 2006), Trypanosomes (Ameisen *et al.* 1995; Duszenko *et al.* 2006; Welburn *et al.* 2006), *Leishmania* (Moreira *et al.* 1996; Zangger *et al.* 2002), and *Plasmodium* (Al-Olayan *et al.* 2002; Arambage *et al.* 2009; Meslin *et al.* 2007; Picot *et al.* 1997). The occurrence of PCD in unicellular parasites has proved controversial because, whilst the morphologies observed are consistent with apoptosis, it appears that the pathways

involved are different to those in mammalian cells where the majority of research has focussed (Debrabant *et al.* 2003; Vercammen *et al.* 2007).

It is likely that as with other eukaryote cells (Degterev and Yuan 2008) various forms of programmed cell death may be important in protozoan parasites including autophagy (Totino *et al.* 2008). The detection of, and semantics for, parasite apoptosis is the focus of other recent papers (Jimenez-Ruiz *et al.* 2010; Kroemer *et al.* 2009; Meslin *et al.* 2011). Here, I use the term 'apoptosis' to describe cells that have made the decision to die (analogous to suicide) as a strategy to improve transmission of surviving parasites. The distinction between apoptosis a strategy employed by parasites to die and apoptosis as simply the way in which parasites die when they are killed by host/vector factors is key; the former predicts apoptosis benefits all parasites in an infection, the latter predicts that a reduction in numbers is detrimental. As I am primarily interested in the evolutionary explanations for apoptosis to occur my focus is on apoptosis as a parasite strategy.

The occurrence of apoptosis in unicellular parasites is a challenge to explain because "Darwinian survival of the fittest" assumes organisms have evolved strategies to maximise their proliferation not their death. Here, I outline possible evolutionary explanations for apoptosis in protozoan parasites and suggest how they should be tested, with an emphasis on the importance of considering parasite ecology. I focus on malaria (*Plasmodium*) parasites as the application of an evolutionary framework to understand parasite life-history traits is better developed for malaria than other protozoan parasite species (e.g. Mackinnon and Marsh 2010; Mackinnon and Read 2004; Paul *et al.* 2003; Reece *et al.* 2009). However, natural selection finds similar solutions to shared problems; therefore, it is likely that my message will be applicable more broadly to protozoan parasites. I start by outlining what is currently known about apoptosis in malaria parasites and the possible evolutionary explanations for why parasites would employ this strategy. I then go on to highlight the ecological factors which should be considered in choosing markers and conducting experiments on protozoan apoptosis, before suggesting possible future directions for testing evolutionary explanations.

5.3. Apoptosis in malaria parasites

Whilst in their vertebrate host's circulation, *Plasmodium* parasites produce asexual stage parasites, which go through rounds of replication within their host's red blood cells, and so maintain the infection. They also produce sexually differentiated transmission stages (gametocytes) which no longer replicate but if taken up by the mosquito vector provide the potential for transmission (Bannister and Mitchell 2003; Ross 1897). When taken up in a vector's blood meal, gametocytes must immediately differentiate into male and female gametes and mate (Figure 5.1.). Within 18-20 hours post fertilisation, each zygote transforms into a motile ookinete, which traverses the midgut wall and invades the epithelium of their vector. Here, each ookinete differentiates into an oocyst and divides asexually to produce thousands of sporozoites. When an oocyst ruptures, its sporozoites are released into the haemocoel to migrate to the salivary glands, ready to be injected into new hosts (Bannister and Mitchell 2003; Beier 1998). This whole process, termed sporogony, takes around 21 days for *P. berghei* in *Anopheles stephensi* (Sinden *et al.* 2007).

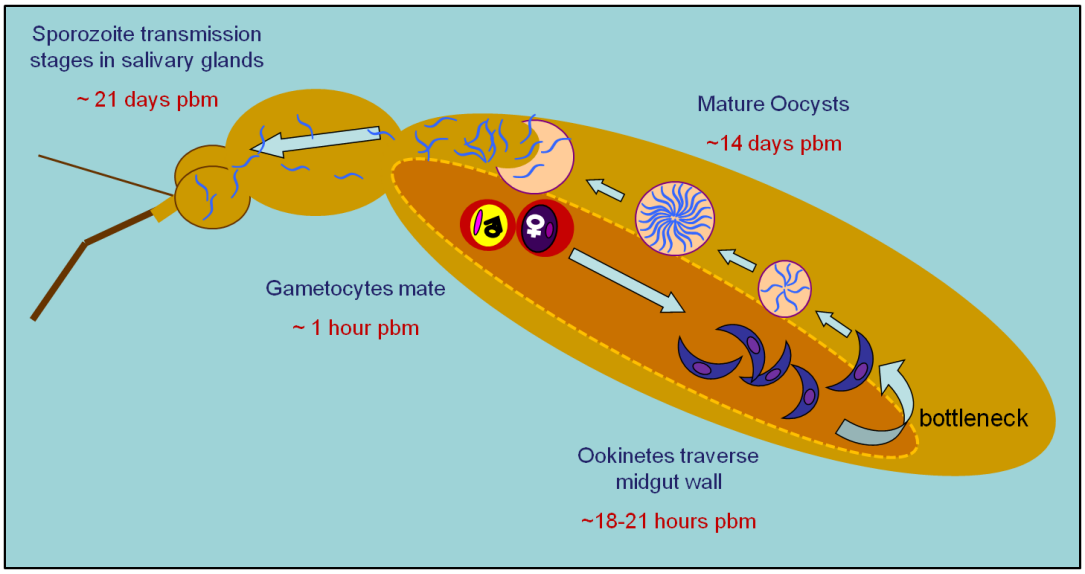

Figure 5.1: Life cycle of malaria parasites within the mosquito vector (sporogeny). Timings show approximate estimates of the stages post blood meal (pbm) for the progression of *P. berghei* though *Anopheles stephensi*. Apoptosis has been observed in three species of malaria parasites at the ookinete stage. The ookinete to oocyst transition is a well known bottleneck in the parasite life-cycle. Estimated reductions in numbers at this point in the life cycle are variable but for *P.berghei* in *A. stephensi* one estimate is a 35 to 120 fold reduction in parasite numbers depending on the density within the vector (Sinden *et al.* 2007).

Recent research has revealed that large numbers of ookinete stage parasites display a variety of apoptosis markers (e.g. condensed chromatin, fragmented DNA, caspase-like activity, translocation of phosphatidylserine and loss of mitochondrial membrane potential; Table 5.1.). This includes observations in the rodent malaria parasite *P. berghei* (Al-Olayan *et al.* 2002), as well as the human malaria parasite *P. falciparum* (Arambage *et al.* 2009). There is also evidence of apoptosis markers in zygotes of *P. berghei* (Al-Olayan *et al.* 2002) and in asexual stages of *P. falciparum* after treatment with the anti-malarial drug chloroquine (Ch'ng *et al.* 2010; Meslin *et al.* 2007; Picot *et al.* 1997) and a common apoptosis inducer staurosporine (Ch'ng *et al.* 2010). My own data provides further evidence for apoptosis in the ookinete stage of *P. berghei* and also the first evidence for *P. yoelii* ookinetes (Table 5.1.; Figure 5.2.; appendix 5.9). Furthermore, previously published accounts of *Plasmodium* parasites displaying 'crisis' or 'degenerate' forms may provide earlier examples of PCD in

malaria (Barnwell and Desowitz 1977; Jensen *et al.* 1982; Stevenson *et al.* 1989; Taliaferro and Taliaferro 1944; Tiffert *et al.* 2000). Controlled experimental approaches have demonstrated that this phenomenon occurs independently of mosquito and host immune cells and is not unique to *Plasmodium* parasites; evidence for apoptosis across a range of protozoan parasites (including *Leishmania, Trypanosoma* and *Toxoplasma* spp.) is rapidly accumulating (Ameisen *et al.* 1995; Gonzalez *et al.* 2007; Moreira *et al.* 1996; Zangger *et al.* 2002).

Protozoan parasites cause some of the most serious infectious diseases of humans, livestock, wildlife, and companion animals. The discovery that these unicellular organisms undergo apoptosis, and that the underlying molecular and cellular processes appear to differ from those of multicellular eukaryotes, offers a potential new paradigm for medical and veterinary interventions (Meslin *et al.* 2007). However, these differences have also resulted in controversy over which methods, terminology, and markers are appropriate for protozoan parasites. This debate must be resolved before parasite apoptosis can be understood: from the proximate genetic, molecular and cellular mechanisms that orchestrate cell death, to the ultimate evolutionary explanations for the existence of PCD in unicellular organisms.

Table 5.1: Variation in rates of apoptosis and temporal patterns observed in malaria parasites. Results organised by life-cycle stage and study. Although the majority of studies summarised here have focused on the ookinete parasite stage, there is still considerable variation in the proportion of cells judged to be apoptotic. This variation may be due to differences in experimental set up between labs, e.g. the nutrients available to parasites and the densities of cultures. Assays of apoptosis may also vary at specific time points in the proportions of positive cells depending on the time scale of the processes being assayed. When parasites are assayed over a time course there is a general trend for an increase in positive cells with time.

Sp	stage	ref	condition	Marker	Detection method	Positive (%)
P. b	Ook	a.	*In vitro* in PBS suspension or **In RPMI**	Condensed chromatin	Acridine orange *	18 hrs – 15.5 (±1.06) **18 hrs – 34.5 (±1.76)** **22 hrs – 55.8 (±13.68)** **26 hrs – 49.01 (±5.51)**
				Fragmented DNA	TUNEL **	**18 hrs – 48.55 (±6.01)** **22 hrs – 64.19 (±6.09)** **26 hrs – 69.89 (±2.81)**
				Caspase-like activity	CaspaTag ***	18 hrs – 17.0 (±2.12) **18 hrs – 30.15 (±2.14)** **22 hrs – 43.8 (±1.53)** **18 hrs – 47.72(±3.93)**
				Translocation of phosphatidylserine	Annexin V-FITC *	**18 hrs – 19.57 (±1.88)** **22 hrs – 28.33 (±5.61)** **26 hrs – 30.12 (±2.75)**
				Mitochondrial membrane potential	JC-1 assay kit****	18 hrs – 34.38 (±2.95)
	Ook	b.	*In vitro* **In RPMI**	Condensed chromatin	Acridine orange *	**24hrs - 31** **36hrs – 80**
	Ook + Gam	b.	*In vivo*	Condensed chromatin	Acridine orange *	18, 20 & 24 hrs – all over 60
	Ook	c.	*In vitro*	Translocation of phosphatidylserine	Annexin-FITC *	< 3 (assay time not reported)
				Fragmented DNA	ApopTag® Fluorescein ***	No positive cells observed (assay time not reported)
				Condensed chromatin	Acridine orange *	No positive cells observed (assay time not reported)
				Caspase-like activity	CaspaTag ***	**21 hrs – 3.8 (±0.05)** **24 hrs – 14 (±9.00)**
	Ook	d.	*In vitro* **In RPMI**	Caspase-like activity	CaspaTag ***	**15 hrs – 13.70 (±12.20)** **18 hrs – 13.06 (±6.42)** **21 hrs – 45.90 (±11.00)** **24 hrs – 67.94 (±4.83)**
	Ook	d.	*In vitro* **In RPMI**	Fragmented DNA	TUNEL *****	**15 hrs – 9.38 (±4.44)** **18 hrs – 14.57 (±3.29)** **21 hrs – 22.08(±8.96)** **24 hrs – 9.24 (±3.09)**
	Ook	e.	*In vitro* **In RPMI**	Caspase-like activity	CaspaTag ***	**18 hrs – 20.06 (±3.50)**

Table 5.1: Variation in rates of apoptosis and temporal patterns observed in malaria parasites (continued)

P. y	Ook	d.	*In vitro* **In RPMI**	Caspase-like activity	CaspaTag ***	**15 hrs – No positive cells observed** **18 hrs – 4.85 (±1.40)** **21 hrs – 62.8 (±11.10)** **24 hrs – 92.59 (±7.41)**
	Ook	d.	*In vitro* **In RPMI**	Fragmented DNA	TUNEL *****	**15 hrs – 7.29 (±3.84)** **18 hrs – 7.41 (±4.90)** **21 hrs – 6.09 (±2.92)** **24 hrs – 9.70 (±0.36)**
P. f	Ook	a.	*In vivo*	Fragmented DNA	TUNEL **	24hrs - 67.8 (±2.82)
	Asex	f.	*In vivo* + CQ	Loss of mitochondrial transmembrane potential	Carbocyanine dye JC-1	Timings and proportions positive not reported
				Fragmented DNA	TUNEL *****	
		g.	*In vivo* + CQ	DNA laddering	$	Timings and proportions positive not reported
P. f	Asex	h.	*In vivo* + (CQ) or (ST)	Loss of mitochondrial transmembrane potential	$$	10 in untreated cultures increased to 31 (CQ) and 25 (ST)
				Caspase-like activity	CaspaTag ***	10 in untreated cultures increased to 34 (CQ) and 32 (ST)
				Fragmented DNA	TUNEL $$$	10 in untreated cultures increased to 27 (CQ) and 56 (ST)

Ook = ookinetes, Gam = gametocytes, Asex = asexual blood stage parasites, CQ = chloroquine (anti-malarial drug), ST = staurosporine (anti-malarial drug)

Assays: *Sigma, ** histochemical, Calbiochem, UK, ***Chemicon international, USA, ****Molecular Probes, UK, ***** *In situ* cell death detection kit, Flourescein Roche, $ After electrophoresis, Southern blotting and autoradiography, a ladder pattern observed, $$ Cell-permeable lipophillic cation probe JC-1 (Molecular probes, Eugene, USA), $$$ ApoDirect DNA fragmentation assay kit (Clontech, San Diego, USA)

References: a. Arambage *et al.* 2009 b. Al-Olayan *et al.* 2002 c. Le Chat *et al.* 2007 d. data reported here (figure 5.2) e. data reported here (figures 5.4 & 5.5) f. Meslin *et al.* 2007 g. Picot *et al.* 1997 h. Ch'ng *et al.* 2010.

5.4. Evolutionary explanations for apoptosis

For unicellular parasites, suicide may appear to be a counter-intuitive strategy when organisms are expected to have evolved to maximise their proliferation. However, uncontrolled replication often is not the best strategy for parasites as this may lead to the host or vector dying before there is a chance for transmission (Poulin 2007). Evolutionary theory therefore suggests that under certain conditions parasites will be selected to display prudence in order to prevent premature death of their host, maximising the time and resources available for transmission and therefore their

fitness (Ebert and Herre 1996; Ewald 1983; Poulin 2007). Apoptosis in single celled organisms can be viewed as an extreme form of prudence and a cooperative (helping) behaviour.

It has been suggested that protozoan parasites may undergo apoptosis as a cooperative behaviour in order to prevent killing the host/vector (Al-Olayan *et al.* 2002; Duszenko *et al.* 2006; Hurd and Carter 2004; Welburn and Maudlin 1997; Welburn *et al.* 2006). However, evolutionary theory predicts that a cooperative behaviour will only evolve if the cost to the individual performing the behaviour (the actor) is outweighed by the benefit to the recipients weighted by the relatedness of the actor to the recipients (Hamilton 1963; Hamilton 1964). A parasite committing suicide (via apoptosis) is an extreme form of cooperation, and obviously death is the highest cost payable. This means that the relatedness between apoptotic parasites and the survivors must be high, and that there must be a substantial benefit provided to the survivors, otherwise a trait as costly as suicide could not have evolved. If infections are initiated by one or very few clones then relatedness will be high. In this case, the question becomes 'how can overall transmission success (fitness) be improved by a reduction in parasite density?' For malaria parasites, there are at least two non-exclusive reasons why lowering parasite density could increase the chance of successfully completing the life cycle in the vector and being transmitted.

First, capping the density of ookinetes within a mosquito may prevent premature mortality of the vector by limiting damage caused either by ookinetes traversing the midgut epithelium or by later stages in sporogony (Hurd *et al.* 2005). Limiting damage to mosquitoes may be particularly important for malaria parasites as the development time required to be transmissible from the salivary glands (~3 weeks) is long compared to the average life expectancy of mosquitoes in the wild, which some estimates put at as low as 1-2 weeks for adult females of the *Anopheles* species (Gwadz and Collins 1996). Therefore, slight variations in the mortality rates of mosquitoes could have a significant effect on parasite transmission. The effect of malaria infection on mosquitoes is controversial with some studies finding a positive correlation between oocyst density and mosquito mortality, but others finding no evidence of a cost to lifespan (reviewed in Ferguson and Read 2002a). These

contrasting results may be due to artificially good conditions in lab experiments masking negative effects of malaria infection (Ferguson and Read 2002a). However, if parasites employ apoptosis to limit damage to mosquitoes then a benign effect of infection should not be surprising. Second, ookinetes at high density could directly effect sporozoite production if oocysts at high density compete for access to limited resources (e.g. nutrients), or indirectly by inducing stronger mosquito immune responses. Little is known about the developmental requirements of oocysts, but malaria infected mosquitoes are more likely to sugar-feed and divert resources away from reproduction (through apoptosis of their ovary cells; Hurd *et al.* 2006; Rivero and Ferguson 2003), suggesting that malaria infection causes a significant energetic burden.

Cooperation is widespread and recent advances in mathematical theory and empirical methods have revealed that the same general principles explain the evolution of cooperation and conflict across a wealth of taxa (from bacteria to insects to humans). This framework predicts that ookinetes will undergo apoptosis when closely related parasites benefit, and ookinete numbers are high enough to negatively affect mosquito lifespan or sporozoite production. These predictions provide the specific, testable, hypotheses that: the proportion of apoptosing ookinetes will: (i) be density dependent and increase with the number of ookinetes in the midgut; and (ii) be greatest when ookinetes in an infection are clonally related and decrease as the genetic diversity of parasites sharing a vector increases (see appendix 5.10). Examples of unicellular parasites cooperating with relatives in a density-dependent way are common (West *et al.* 2007; West *et al.* 2006). Many of the best examples of this come from bacteria that form complex structures called biofilms to provide protection from the host immune response or antibiotic drugs (Webb *et al.* 2003), and bacteria that forage cooperatively to extract iron in a usable form from their host (Buckling *et al.* 2007).

Despite the generality of the evolutionary principles that explain cooperation, the suggestion that apoptosis in malaria parasites is a social trait is controversial. Whilst in bacteria, quorum sensing mechanisms have been described to explain the coordination of behaviour (Diggle 2010; Swift *et al.* 2001), as yet, no specific

quorum signalling system has been found for malaria parasites. However, evidence that malaria parasites respond to changes in their within-host environment by altering their resource allocation decisions show that they can detect and respond to factors such as the presence of competitors and variation in resource availability (Pollitt *et al.* 2011b; Reece *et al.* 2008; Reece *et al.* 2009). The predictions for why parasites undergo suicide are clear and testing them will resolve whether parasite apoptosis has been shaped by natural selection to enable parasites to cooperate with their kin. As with all emerging and interdisciplinary fields, undertaking the key, conceptually simple experiments required to test these predictions is constrained by the limitations of the methods and techniques available. For example in bacteria targeted disruptions have been useful in testing the fitness consequences of specific phenotypes (e.g. Jiricny *et al.* 2010), however, specific candidate genes for apoptosis in malaria are lacking and complex traits are difficult to disrupt. In the next section I outline the methodological constraints that currently impede the collection of data of high enough quality to undertake quantitative tests of the evolutionary explanations for parasite apoptosis. Given the medical and economic implications of malaria parasites and the drive to develop transmission-blocking intervention strategies, understanding their transmission biology from an evolutionary perspective is also timely and important.

5.5. Ecological considerations: applying assays for morphological markers

I suggest that to examine the evolutionary causes and consequences of apoptosis in malaria parasites, their ecology must be taken into account when deciding which assays to use and how best to apply them. In Table 5.2 I outline the markers and assays available, highlighting their suitability (from an ecological perspective) for use with malaria parasites, and I discuss the general issues below.

Table 5.2: Summary of some commonly used markers for apoptosis. Particular reference is paid to ecological and practical considerations when using to test evolutionary predictions. For more details on the markers of apoptosis in protozoan parasites see Jimenez-Ruiz *et al.* (2010).

Marker	Method of detection	Practical considerations	Relevance for malaria ecology
Activation of caspase-like molecules e.g. CaspaTag *	A fluorescent labelled general caspase inhibitor (FAM.VAD.fmk (green) or SR.DEVD.fmk (red) bind to active caspase within the cell. Ve+ cells display fluorescence	-Quick and easy to use -Results not as clear as TUNEL -Performed in conjunction with viability tests -Large scale experiments possible -The caspase inhibitor is broad spectrum and may cross-react with unrelated molecules.	The role of caspase-like molecules is controversial in protozoan parasites, therefore it is not possible to be certain that apoptosis is being detected. However, if caspase molecules are a reliable marker they would be useful as an early marker of induction.
Depolarisation of mitochondria outer membrane e.g. JC-1 assay kit **	JC-1 (cationic carbocyanine dye) accumulates in mitochondria. Emission of fluorescence shifts from orange (polarised mitochondrial membrane) to green (depolarised mitochondrial membrane).	-Quick and easy to use - Performed in conjunction with viability tests	The role of mitochondria in malaria apoptosis not well established. However, if markers prove to be reliable they would be useful as an early marker of induction.
Condensed chromatin e.g. Acridine orange ***	Differentially stains SS and DS nucleic acids - showing condensed chromatin. Ve+ cells display intense red staining in nucleus	-Quick and easy to use -False positives? - Results not as clear as with TUNEL - Performed in conjunction with viability tests -Large scale experiments possible	Good relevance - this process is predicted to be the same for mammalian and protozoan cells.
Translocation of phosphatidylserine to outer cell membrane e.g. Annexin V-FITC ***	Ve+ cells display green annexin labelling on the cell surface.	-Quick and easy to use -Results not as clear as with TUNEL - Performed in conjunction with viability tests - Large scale experiments possible	May not be relevant for malaria cells for two reasons. 1. The cell membrane of protozoan parasites are very different to mammalian cells. 2. Mammalian cells express phosphatidylserine to be taken up by phagocytes, is not relevant for the mosquito midgut.

Table 5.2: Summary of some commonly used markers for apoptosis (Continued)

Fragmented DNA leading to the generation of fragments with 3'OH groups ****	DNA of fixed and permeabilized cells labelled by the addition of flourescein dUTP at strand breaks by terminal transferase. Ve+ cells display fluorescent nucleus (figure 3)	- More laborious than using Acridine orange, CaspaTag or Annexin V - Clear unambiguous results. - Requires cells to be dead so cannot perform viability tests in conjunction. - Slides can be stored (at 5°C) for later analysis. Therefore large scale experiments possible	Good relevance as process expected to be the same for mammalian and protozoan cells. However as DNA fragmentation is thought to be a late process in apoptosis may only see markers at a later time point than induction of apoptosis pathways. Some necrotic cells may show positive.
Morphological Markers (membrane blebbing and formation of apoptotic bodies)	Observation of cell morphology under electron microscope.	-Time consuming and expensive -Not a good basis for morphological changes seen in malaria apoptosis -Requires cells to be dead so cannot perform viability tests in conjunction. -Large scale experiments not possible but may be useful in conjunction with other assays	Malaria parasite cells differ in structural aspects from mammalian cells, it is therefore not clear whether the structural changes observed in mammalian cells would be relevant for these parasites. The ultimate reasons for formation of apoptotic bodies to be taken up by macrophages also not relevant in the mosquito midgut.
Detecting cell viability			
Propidium iodide (PI) *****	Stain is taken up in cells with compromised membranes. Ve+ cells display red fluorescence.	Quick and easy to use Cells must be viewed quickly after application Can be used in conjunction with assays on live cells e.g. CaspaTag.	Useful method for assessing viability of cells which can be used in conjunction with other assays of apoptosis.

*Chemicon international, USA, **Molecular Probes, UK, *** Sigma, UK, **** *In situ* cell death detection kit, Flourescein (Roche), *****Roche

5.6. Which markers matter?

Many kits for assaying markers of apoptosis in mammalian cells are commercially available. However, it is not clear which markers are most suitable for measuring apoptosis in protozoan parasites. For example, one common marker for apoptosis of mammalian cells is the translocation of phosphatidylserine to the outside of the cell membrane (detected using an annexin assay). In mammals phosphatidylserine provides a signal for the apoptotic cell to be engulfed by phagocyctes, which prevents the cell from disintegrating and the resultant debris from causing inflammation (Savil and Fadok 2000). Whilst such a 'tidy death' is clearly advantageous in a muticellular organism, it may not be an applicable concern for single-celled ookinetes in the mosquito midgut. However, the presence of this marker in yeast and leishmania suggests that it may have additional functions (Fröhlich and Madeo 2000; Tripathi and Gupta 2003). In leishmania it is thought that phosphatidylserine translocation acts as a form of 'apoptotic mimicry' which aids the parasite in infecting macrophages (Tripathi and Gupta 2003). In yeast the reason for the membrane altering during apoptosis is not yet known, however an interesting possibility is that it could act as a signal to other yeast cells.

Another common marker for apoptosis is caspase activity. In mammalian cells, classical apoptosis is triggered by the activation of caspases, which are apoptosis-specific cysteine proteases within the clan CD (Hengartner 2000; Rawlings *et al.* 2002). Part of the controversy surrounding the characterisation of parasite apoptosis stems from the absence of 'true' (canonical) caspases in protozoans (Atkinson *et al.* 2009; Vercammen *et al.* 2007). However, ancient caspase homologues (known as metacaspases) are present in the genomes of plants, fungi and protozoa (Uren *et al.* 2000). In plants and fungi metacaspases have been shown to be involved in apoptosis (He *et al.* 2008) and four metacaspases have been identified in the *Schistosoma mansoni* and *S. japonicum* (Atkinson *et al.* 2009; Berriman *et al.* 2009) which could also play a role in a form of PCD. In *Plasmodium* there are three metacaspases (PxMC1, PxMC2 and PxMC3), and like the mammalian counterparts, they all posses a defined pro-region and a catalytic domain that is indicative of the clan CD, family C14 caspases. It has been suggested that one or more of these metacaspases can carry

out a functionally analogous biological role to metazoan caspases, and although data are scarce, they have been linked to programmed cell death in some unicellular organisms (Gonzalez *et al.* 2007; Kosec *et al.* 2006a; Kosec *et al.* 2006b; Madeo *et al.* 2002b). Of the three metacaspases in *Plasmodium*, MC1 may represent the best candidate enzyme to be involved in a form of PCD in *Plasmodium* as it is the only MC that has the required predicted catalytic cysteine and histidine residues in the correct context for an active enzyme, and is typified by the *P. falciparum* enzyme PfMC1 (PF13_0289; Mottram *et al.* 2003; M. Sajid personal communication) However, it is now evident, from work on mammalian cells, that beside the caspase dependent form of apoptosis a caspase independent form can also occur (Deponte 2008; Leist and Jäättelä 2001).

The role of metacaspases in protozoan apoptosis is controversial, and contrasting conclusions have been drawn from different experiments (Deponte 2008; Váchová and Palková 2007; Vercammen *et al.* 2007). Supporting data come from studies showing that the addition of the broad spectrum caspase inhibitor Z-VAD.fmk results in a reduction of the number of ookinetes displaying a variety of apoptotic morphologies, and also a doubling in the number of oocysts in the mosquito midgut (Al-Olayan *et al.* 2002). In contrast, one study examining a *P. berghei* line in which PbMC1 has been deleted, found no loss of apoptosis (judged by CaspaTag) and therefore concluded that it may be a functionally redundant gene (Le Chat *et al.* 2007). However, these authors did not apply any other markers to their knock out line and found very low rates of phosphatidylserine translocation and no DNA fragmentation or chromatin condensation with their wild-type line. My own research has shown that when compared to genetically intact parasites, a PbMC1 knock out line reaches higher ookinete and oocyst densities in infected mosquitoes and that this translates into lower sporozoite production and higher mosquito mortality. This demonstrates there is a cost to sporogony at high parasite densities and suggests that PbMC1 mediates ookinete numbers although it is not yet clear if this is through apoptosis (chapter 6).

Recent research has implicated clan CA cysteine proteases in chloroquine mediated apoptosis (Ch'ng *et al.* 2010). However, the use of inhibitor/probes developed for use in humans or other systems with canonical caspases should be viewed with caution. To date there are no proteases from Plasmodium that have a specificity for Asp at P1 (as per Val-Ala-Asp (P3-P2-P1)) of the caspase selective probes. This together with irreversible nature (fmk) of these probes is likely to lead to off target inhibition and the clan CA may be amongst these off target hits (Rozman-Pungercar *et al.* 2003). This problem is compounded if these inhibitors are used either at very high concentrations or they are used over an extended time period.

5.7. How should assays be applied?

One of the attractions of using the activation of caspase-like molecules as a marker for apoptosis is the ability to assay cells in the early stages of apoptosis. This is important for studies aiming to assay apoptosis at biologically relevant time points. For example, ookinetes begin to develop from retort (immature) forms at around 8 hours post fertilisation and begin to invade the gut epithelium at 18-21 hours for *P. berghei*, although these timings vary between malaria parasite species (Beier 1998). If some ookinetes undergo apoptosis in order to provide a benefit to others, apoptosis is expected to be initiated before 18-20 hours post fertilisation. Therefore, it is important to assay apoptosis rates before ookinetes have either died or transformed to the next life cycle stage. Assays for depolarisation of mitochondrial membrane potential may also have the advantage of being an early marker of apoptosis (Kroemer *et al.* 1998; Ly *et al.* 2003), however as with many of assays discussed here the relevance for malaria parasite cells has not yet been established. Intuitively, assaying the activation of death executors would appear to be the best approach when testing whether levels of apoptosis are linked to developmental schedule but early apoptotic mammalian cells can be saved (Hengartner 2000) and it is not yet known at what stage parasites become irreversibly committed to dying.

A possible solution to this problem is to assay morphologies observed at the end of the apoptosis program, such as DNA fragmentation. However, this approach may complicate the ambition of examining apoptosis in a biologically relevant timeframe. The key question is how long does the apoptosis program in malaria parasites take?

For example, if ookinetes are predicted to initiate apoptosis at 18 hours post fertilisation, how much later should DNA fragmentation be assayed? I show a preliminary examination of this issue in Figure 5.2. The proportion of cells with fragmented DNA at the time points measured are significantly lower than those displaying caspase-like activity, but clearly more studies are required to characterise the time lags between the activation of apoptosis and the appearance of the resulting morphologies. These studies should be designed with reference to the developmental schedules of parasite species that undergo sporogony at different temperatures. Another problem with assaying late-stage apoptosis morphologies is the validity of combining assays to distinguish between apoptotic cells and necrotic cells. Furthermore, any temporal variation in how individual parasites initiate and progress through apoptosis will make it difficult to distinguish between cells in a sample that are healthy, undergoing apoptosis, have died by apoptosis, or have died by necrosis. This could be further complicated by the fact that assays such as TUNEL for DNA fragmentation can also be positive in necrotic cells. This all points to the importance of having reliable time-lines for the changes associated with the different forms of cell death.

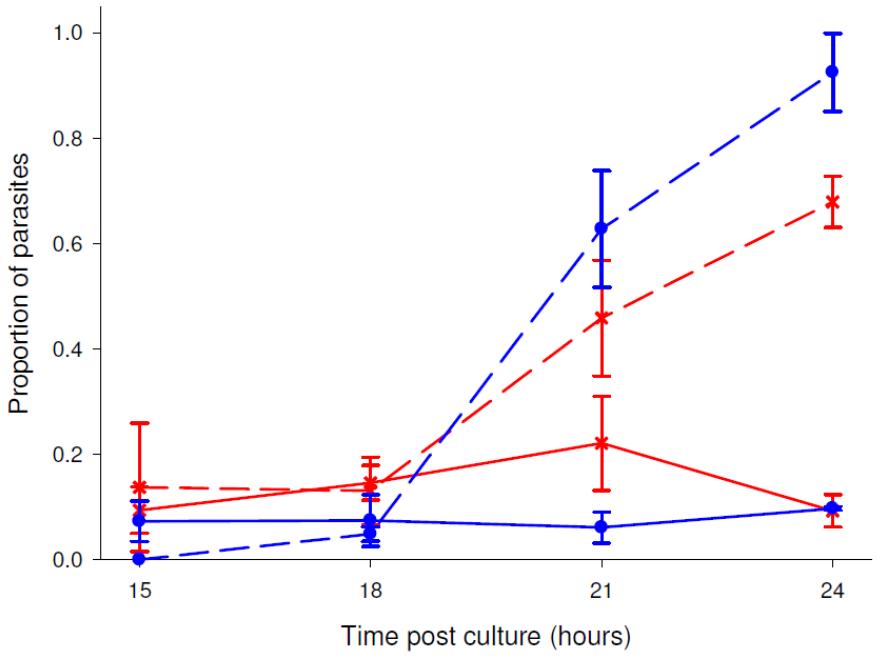

Figure 5.2: Markers for apoptosis vary over time and between species. Graphs show the proportion of ookinete stage parasites displaying DNA fragmentation as measured by TUNEL (solid lines) and Caspase-like activity (dashed lines) as measured by CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Fluorescein in conjunction with propidium iodide (Chemicon international, USA) in *Plasmodium berghei* (crosses) and *Plasmodium yoelii* (circles). Bars show standard errors of the mean and values are based on between 3 and 6 individual infections per time point. For each mouse 8 ookinete cultures were set up which were incubated for 14 hours before the cultures from each mouse were pooled and purified for ookinetes using macs ls cell separation columns (Miltenyi biotec). The resulting purified ookinetes were then alliquated into 8 individual 1ml cultures (1 per time point for 2 assays) containing complete ookinete media. These cultures were then returned to the incubator until the time relevant time point (15, 18, 21 or 24 hours post culture set up). More detailed methodology is available in appendix 5.9.

In my experience, measuring DNA fragmentation by fluorescent TUNEL assay appears to provide repeatable and non-subjective results (positive cells show an obviously fluorescing nucleus; Figure 5.3). Fragmentation of DNA is also a well defined end point to a program of programmed cell death. However, because TUNEL assays are applied to fixed and permeabilized cells, parasites that do not display DNA fragmentation cannot be further characterised as healthy or necrotic. For this reason, assays that can be applied to live parasites are very useful, such as CaspaTag (but see 'which markers matter' section). Because most apoptosis assays have been developed for mammalian cells, the protocols involved may not always be appropriate for live parasites. For example ookinetes, which develop at considerably lower temperatures, would experience considerable stress and heat shock if treated at 37ºC during assaying. Electron microscopy on parasite nuclei can be useful to verify and compare processes in order to reliably discriminate between apoptosis and other forms of programmed cell death. However, this is not practical for hypothesis testing on large numbers of cells. For most experiments wanting to study ecological variation then it is also necessary that assays allow high through-put of samples. In these situations assays on dead and fixed parasites that can be stored for later analysis (e.g. fluorescent TUNEL) are more practical. In addition to studies that characterise parasite apoptosis programs, technical developments are required so that assays can be applied to large numbers of cells and enable their morphologies to be efficiently and accurately quantified.

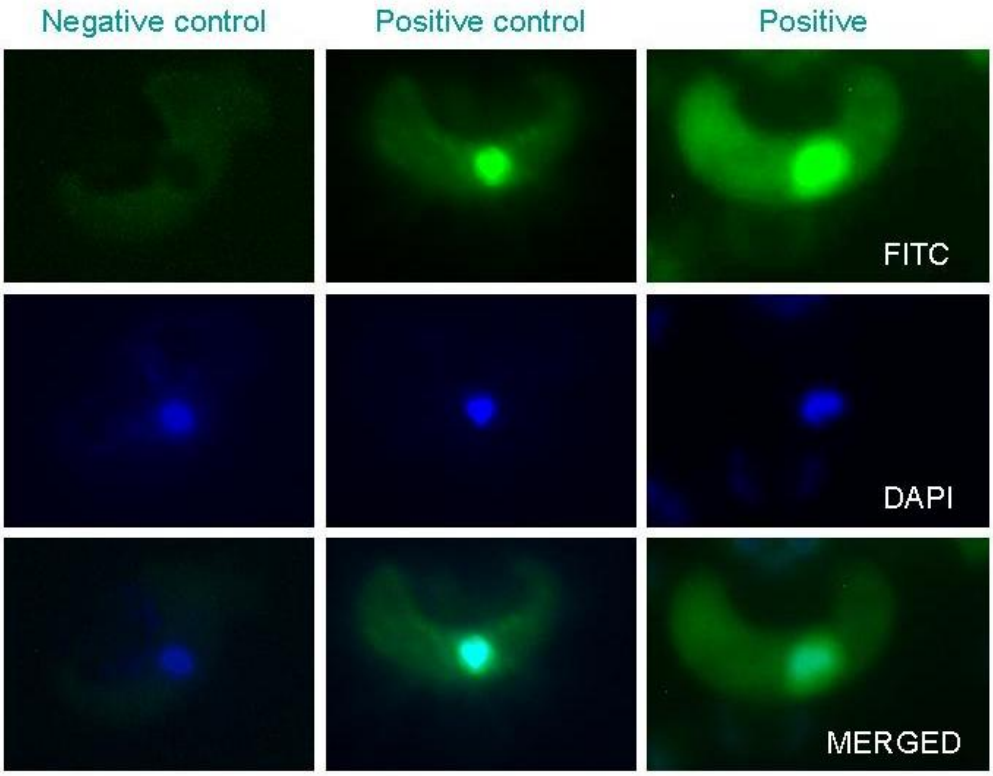

Figure 5.3: Identification of DNA fragmentation by fluorescent TUNEL assay. Images of *P. berghei* ookinetes with fragmented DNA marked using a TUNEL assay (*In situ* cell death detection kit, Fluorescein, Roche) at 21 hours post culture. Positive cells show a bright green nucleus. Negative controls were incubated with only the label solution without the enzyme and positive controls had DNA strand breaks induced with DNase 1 recombinant (3 units/ml for 10mins at 20°C) before labelling (DNase 1 recombinant, grade 1, Roche). DAPI staining was used to check the location of the nucleus. More detailed methodology is available in appendix 5.9.

5.8. Variation: noise or not?

A major challenge for evolutionary biology is explaining variation in traits observed across genotypes, and within the same genotype in different environments. The relative proportions of parasites recorded as undergoing apoptosis varies over time, between markers and across studies (see Table 5.1). This variation is common in evolutionary studies of phenotypic traits and initially seems difficult to interpret. The challenge is to identify patterns and understand what is driving them. Recent studies have revealed that malaria parasites detect and respond to subtle changes in the conditions they encounter during infections by altering traits such as investment in gametocytes and their sex ratio (Paul *et al.* 2000; Pollitt *et al.* 2011b; Reece *et al.* 2010; Reece *et al.* 2008). These conditions include the density of clone-mates and genetic diversity of co-infecting parasites, which are also the factors predicted to influence levels of apoptosis. Therefore, variation across studies in infections and / or experimental set up may result in differences in the cues that parasites experience or their ability to detect this information.

My data show that even within controlled replicate infections – initiated with the same infective dose of the same parasite clone in the same batch of hosts – there is variation in the proportion of parasites displaying markers of apoptosis (Figure 5.4). This may be due to parasites responding to subtle variation in parasite densities or other aspects of their within-host environment, such as immune challenge or anaemia. However, when the data from individual infections within an experimental group are combined and two replicate experimental groups are compared, individual replicates are noisy but patterns are consistent (Figure 5.5). This suggests that it is possible to reliably detect patterns but large sample sizes (number of independent infections) and standard conditions are required. Also, where possible, variables such as parasite density should be recorded in order to control for its potential influence on rates of apoptosis.

Parasite ecology may also be important for understanding variation in apoptosis behaviour between different parasite species. For example, a comparison of our data for rates of apoptosis observed in *P. berghei* and the related rodent malaria species *P. yoelii* (Figure 5.2) reveals significantly different patterns of caspase-like expression

over time (species*time χ^2_1=4.38, p=0.046). The proportion of parasites assaying positive with TUNEL was not significantly different over time (χ^2_1=0.001, p>0.5). However the proportion positive at 21 hours was significantly lower in *P. yoelii* (t=2.37, df=8, p=0.045). Given the debate over the reliability of using caspase-like activity as a marker of apoptosis in *Plasmodium*, I propose that a more accurate picture is gained from focussing on the TUNEL positive cells. Can differences in species ecology explain the difference in levels of DNA fragmentation in these species? A possible explanation is that as *P. berghei* parasites can reach considerably greater oocyst densities than *P. yoelii*, higher rates of apoptosis are required for *P. berghei* survivors to gain a benefit in terms of increased transmission success. With this in mind it would be interesting to examine the rates of apoptosis in other malaria parasite species.

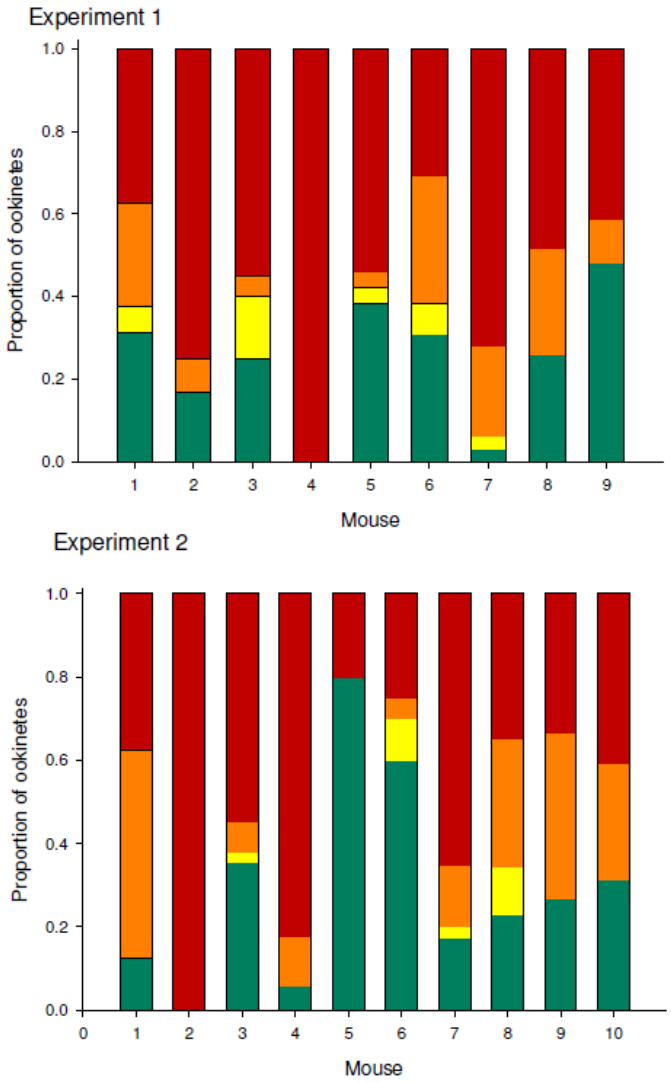

Figure 5.4: Replicate experimental infections show variation for proportions of parasites showing caspase-like activity and viability. Two sets of replicate experiments were set up by infecting 10 male MF1 mice 8-10 weeks old per replicate with 10^7 *P. berghei* parasites after pre-treatment with Phenylhydrazine 2 days pre-infection (120mg/kg). One mouse from experiment 1 failed to become infected so was removed from the study. Cultures were then set up 4 days post infection. After 18 hours ookinetes were purified using MACS LS cell separation columns (Miltenyi biotec) and a minimum of 30 parasites were assayed for caspase like activity and viability using CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Fluorescein in conjunction with propidium iodide (Chemicon international, USA). Green indicates healthy ookinetes negative for caspase-like activity with intact membranes, yellow indicates early apoptotic ookinetes displaying caspase-like activity with intact membranes, orange indicates late apoptotic ookinetes displaying caspase-like activity but also compromised membranes and red indicates dead cells with compromised membranes (Arambage *et al.* 2009). More detailed methodology is available in appendix (5.10).

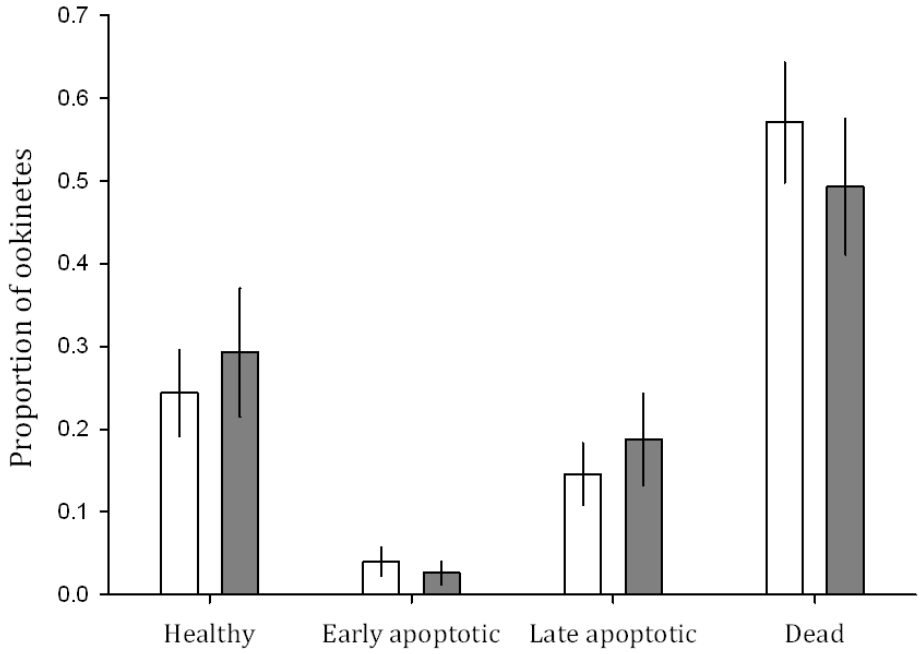

Figure 5.5: Between experiments markers of apoptosis and death are repeatable. Data taken from the experiments described for figure 5.4. Average proportion of ookinetes classified into 4 categories (healthy, early apoptotic, late apoptotic or dead) across 9 replicate infections for experiment 1 (open bars) and 10 replicate infections for experiment 2 (filled bars). Although there is variation for replicate infections within experiments (figure 4) there is no significant variation between the cells categorised into each condition between 2 experiments carried out on different days ($\chi^2 = 5.81$ (3 df), $p>0.1$). Error bars show the standard error of the mean.

5.9. Conclusions

The discovery of apoptosis-like cell death in single celled protozoans such as malaria parasites provides an exciting challenge for evolutionary biology to explain and a new direction for intervention strategies. Progress in both of these fields requires evolutionary biologists to work together with cell biologists to develop reliable high throughput assays to study variation in apoptosis in response to the key parameters of parasite density and infection genetic diversity. At the same time, debates on the best markers for assaying apoptosis and appropriate terminology need to be resolved in order for research to move forward.

5.10. Appendix I: Materials and methods

5.10.1. Infections and culturing parasites

I used the rodent malaria parasites *P. berghei* 820 (Pb820cl1m1cl1 (RMgm-164); Ponzi *et al.* 2009) and *P.yoelii* wild type (WHO Registry of Standard Malaria Parasites held at The University of Edinburgh). For *P.berghei* infections were initiated with 10*7 parasites in male MF1 mice (8-10 weeks old) which had been pre-treated with Phenylhydrazine (PHZ) (120mg/kg 4 days prior to infection). This treatment stimulates the erythropoietic response in the mice encouraging the establishment of the malaria infection and the production of gametocytes (Gautret *et al.* 1996). On day 4 post infection ookinete cultures were set up with 75µl of infected blood in 5mls of complete ookinete culture media (RPMI + 10% fetal calf serum, pH 8) and incubated at 21°C. For *P.yoelii* parasites were culturing conditions were altered in the following ways; Mice were given 60mg/kg of PHZ 3 days prior to infection, blood was collected on day 3 post infection and ookinetes were cultures at 24°C. After incubation ookinete cultures for both species were purified using MACS LS cell separation columns (Miltenyi biotec).

5.10.2. Detection of Caspase-like activity

Caspase-like activity was measured using CaspaTag™ Pan-Caspase In Situ Assay Kit, Fluorescein iodide (Chemicon international, USA) following the manufacturer's instructions except for modification of incubation temperature which were changed from 37°C to 24°C or 21°C respectively for *P.yoelii* and *P.berghei* cells. Previous studies have shown that lowering the incubation temperature to a suitable level for ookinete development does not alter the proportion of cells showing caspase-like activities and that higher temperatures result in increased death in ookinetes as measured by membrane viability (Arambage *et al.* 2009). CaspaTag utilises a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase (FAM-VAD-FMK) which acts as a non-cytotoxic fluorescent broad-spectrum caspase inhibitor that binds covalently to active caspases in living cells. After incubation cells which contain the bound reagent (indicating caspase-like activity) will glow green when analysed by fluorescence microscopy.

5.10.3. Detection of fragmented DNA

DNA fragmentation was measured using TUNEL In situ cell death detection kit, Fluorescein (Roche) following the manufacturer's instructions. Purified ookinete cultures were centrifuged, smeared and fixed onto a glass slide with 4% Paraformaldehyde in PBS (pH 7.4, 1 hour incubation at 15-21°C). Cells were then permeabilised using 0.1% Triton X-100 in 0.1% sodium citrate (2 minutes on ice). Fixed permeabilised cells where then incubated with the TUNEL reaction mixture which labels DNA strand breaks, by terminal deoxynucleotidyl transferee (TdT), which catalyzes polymerization of labelled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL-reaction). Flourescien labels incorporated into the nucleotide polymers are can then detected by fluorescence microscopy (the nucleus of positive cells fluoresce bright green, see figure 5.3.). After incubation with the tunnel reaction mixture VECTASHIELD mounting medium with DAPI (vector laboratories) was added to samples to prevent the fluorescence fading and allow the location of the nucleus to be confirmed.

5.10.4. Detection of cell viability

Cell viability was measured using propidium iodide (PI; 250µM/mL; Chemicon international, USA) which enters cells with permeabilised membranes resulting in red fluorescence.

5.11. Appendix II: Evolutionary predictions for apoptosis in malaria parasites

5.11.1. Model assumptions

We built a model examining the conditions under which apoptosis may have been favoured by selection. We examine how the evolutionarily stable proportion of ookinetes transforming to oocysts will change when the relatedness (*r*) and density of the ookinetes (*N*) in the midgut vary. For the purposes of the model various assumption were made.

I. The process of apoptosis was initiated by the cell itself or clone mates.
II. The number of oocysts positively correlates mortality (Lyimo and Koella 1992; chapter 6).
III. Malaria parasites are able to determine the genetic relatedness of the infection (Reece *et al.* 2008).

5.11.2. Model details

The fitness *(w)* of an individual ookinete depends on the probability of it becoming an oocyst *(x)*, the number of other ookinetes in the midgut *(n)* and the mean probability of transforming to oocysts *(y)*.

$$W(x,y=ny)=xP(ny)$$

Where *(ny)* is the total number ookinetes from the group. Using the methods developed by (Taylor and Frank 1996) we can determine that the ESS for transformation satisfies:

$$J(z^*,n,r)=P(nz^*)+nz^*P'(nz^*)r=0$$

Where r=dy/dx

We now examine the impact of ookinete number on *(n)* on the ESS transformation strategy and rewrite the above in terms of the ESS number of transformers, N*=nz*

$$K(N^*,r) = P(N^*)+N^*P'(N^*)r=0$$

Note that, because dK/dn = $\partial K/\partial N^* \times dN^*/\partial n = 0$, where $\partial K/\partial N^* \neq 0$, it follows that $dN^*/\partial n = 0$. This gives us prediction 1:

Prediction 1: The ESS number of oocysts reaches saturation with the number of ookinetes (figure 5.6a).

From this it follows that the proportion transforming (z*) will decrease with n, giving us Prediction 2:

Prediction 2: The ESS proportion of ookinetes transforming to oocysts will decrease with the number of ookinetes. Therefore, the number of apoptotic ookinetes will increase with their density (figure 5.6b).

We then examine the effect of relatedness on the ESS transformation strategy. In order for the ESS z^* to be a continuously stable strategy (CSS) (Eshel 1983; Taylor 1996) then $\partial J/\partial z^* < 0$ (Pen 2000). Hence, assuming convergence stability, the sign of dz^*/dr is the same as that of $\partial J/\partial r$ (Christiansen 1991), i.e.

$$\frac{\partial J}{\partial r} = nz^* P'(nz^*) < 0$$

This gives us Prediction 3:

Prediction 3: The ESS proportion of ookinetes transforming into oocysts will decrease with genetic relatedness *(r).* Therefore, there will be lower levels of apoptosis in mixed-genotype *(r<1)* infections (figure 5.6c).

Finally, because the ESS optimises group transmission success when $r = 1$, and because z^* increases as r decreases from 1, we have Prediction 4:

Prediction 4: For all genetically heterogenous infections *(r<1)*, the ESS proportion of transforming ookinetes is greater than optimal for transmission (figure 5.6c).

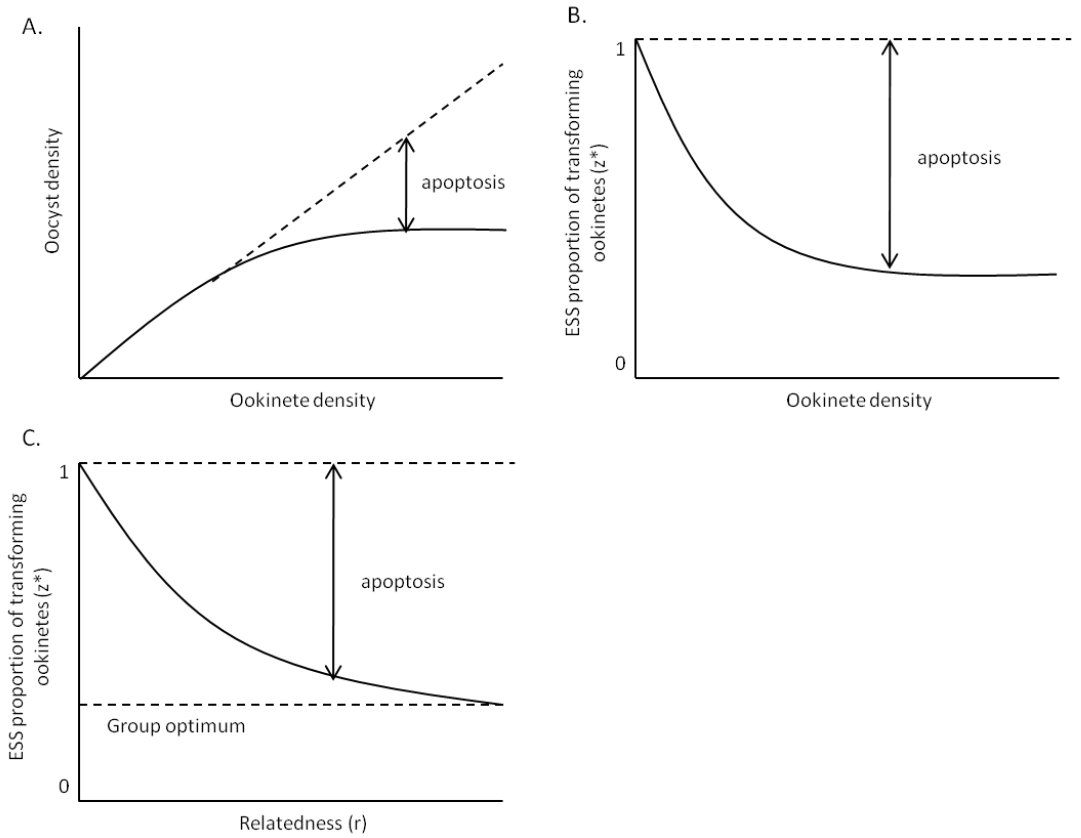

Figure 5.6. Evolutionary predictions for apoptosis in malaria parasites. A. The ESS number of oocysts reaches saturation with the number of ookinetes. B. The ESS proportion of ookinetes transforming to oocysts will decrease with the number of ookinetes. Therefore, the number of apoptotic ookinetes will increase with their density. C. The ESS proportion of ookinetes transforming into oocysts will decrease with genetic relatedness *(r).* Therefore, there will be lower levels of apoptosis in mixed-genotype *(r<1)* infections and for all genetically heterogenous infections *(r<1)*, the ESS proportion of transforming ookinetes is greater than optimal for transmission.

CHAPTER 6

COSTS OF CROWDING FOR THE TRANSMISSION OF MALARIA PARASITES

6.1. Summary

In recent years there has been increasing interest in understanding the dynamics of infectious disease from an evolutionary perspective. This involves bridging scales from within to between host processes and for vector borne parasites, such as malaria, understanding parasite dynamics and interactions both within the host and within the vector. However, while understanding of within-host processes is increasing, parasite dynamics within the vector and their implications for transmission remain poorly understood. Furthermore, available data often seem contradictory and the answers to basic questions such as, 'Is malaria bad for mosquitoes?', remain unclear. Here, I use the rodent malaria parasite *Plasmodium berghei* to manipulate the density of parasites in *Anopheles stephensi* infected mosquitoes, fed either *ad lib* or with restricted water and glucose, and quantify the impact on two key components of parasite fitness: parasite development and vector mortality. I show that high parasite densities result in significant costs of crowding by reducing both parasite proliferation and vector survival. My data demonstrate that density dependent processes can have significant implications for parasite transmission and should be considered in the context of transmission blocking interventions.

6.2. Introduction

The density of individuals within a shared environment is a key factor in determining fitness and consequently shapes both ecological and evolutionary processes (Bassar *et al.* 2010). Generally it is expected that individuals at high densities will experience higher mortality and lower reproductive success, due to competition for resources, apparent competition (e.g. increased density of shared predators), or direct interference competition (Krebs and Davies 1997). However, complex interactions between these factors and variation in environmental factors means that, even after centuries of attention, and their clear importance for evolutionary and ecological processes, the consequences of density dependent effects on fitness and the evolution of life history traits are poorly understood (Bassar *et al.* 2010; Krebs and Davies 1997).

For parasites, density dependent relationships will have important implications for the transmission of disease (Basáñez and Ricárdez-Esquinca 2001). However, quantifying and explaining these relationships is particularly challenging as fitness will depend not only on the parasites' interactions with other individuals sharing the host (e.g. the density of parasites and the relatedness of co-infecting strains; Foster 2005; West *et al.* 2007) but also on characteristics of the within-host environment (e.g. resource availability and anti-parasitic immune responses; Mideo 2009; Pedersen and Fenton 2007; Reece *et al.* 2009; Thomas *et al.* 2002). For vector-borne parasites, such as trypanosomes and malaria parasites, the above considerations will apply not only to density dependent processes within the vertebrate host, but, also to dynamics within the insect vector. However, while there is some work on the relationship between parasite density in the host and the ability to infect vectors (Barnes and White 2005; Drakeley *et al.* 1999; Schall 2000; Schneider *et al.* 2007), infection dynamics within the vector and their impact on transmission between hosts remain poorly understood. In particular for malaria parasites, there is marked variation in the intensity and prevalence of infection in mosquitoes (Beier *et al.* 1991; Medley *et al.* 1993; Schall 2000; Sinden *et al.* 2007; Tripet *et al.* 2008; Vaughan *et al.* 1994), but the reasons for this variation and the implications for vector survival and patterns of transmission remain unclear.

Despite over a century of research since mosquitoes were identified as malaria vectors (Ross 1897), the impact of infection on vector survival remains unresolved. A meta-analysis by Ferguson and Read (Ferguson and Read 2002a) suggested that in general, mosquito survival is reduced by infection with malaria parasites, and this is supported by recent studies (Aboagye-Antwi *et al.* 2010; Dawes *et al.* 2009a); however, this cost is often not detected (Gamage-Mendis *et al.* 1993; Robert *et al.* 1990) or only under certain conditions (Tripet *et al.* 2008). This is likely to be due, in part, to both laboratory and field studies suffering from various limitations (Tripet *et al.* 2008). While field studies offer important insights into the dynamics of parasites under natural settings and vector-parasite combinations, important factors such as the age of individual mosquitoes and the genetic diversity of parasite infections are often uncontrolled and unknown (Aboagye-Antwi *et al.* 2010; Anderson *et al.* 2000). In contrast, while laboratory transmission studies often use unnatural vector-parasite combinations and miss important interactions with the environment, they have provided a wealth of detailed information on parasite development in the vector (Sinden *et al.* 2007; Sinden 2002). An additional confounding factor in detecting the cost of infection is that protozoan parasites, including malaria parasites, have been observed to undergo apoptosis (a form of programmed cell death; Al-Olayan *et al.* 2002; Duzensko *et al.* 2006; Hurd and Carter 2004; Pollitt *et al.* 2010; Welburn and Maudlin 1997; Welburn *et al.* 2006), and the role of this in infections is poorly understood (Pollitt *et al.* 2010).

Parasite density within the vector will affect parasite fitness and transmission by influencing characteristics of the within-vector environment (Cirimotich *et al.* 2010). Crowding may reduce the ability of parasites to survive and proliferate, owing to resource limitation or high density infections triggering a stronger immune response. Alternatively, high density infections could be costly to the parasite through an increased risk of premature vector mortality (Basáňez *et al.* 1996). For malaria parasites infecting mosquitoes, density dependent processes are likely to occur at various points during their progression through the vector (Sinden *et al.* 2007). Firstly, motile ookinete stages traverse the midgut wall and invade the epithelium, which with high parasite numbers is likely to increase vector mortality due to both direct damage to the midgut and the potential for secondary bacterial infections

(Dimopoulos *et al.* 2002; Meister *et al.* 2009). Secondly, after invading the epithelium the ookinetes differentiate into oocysts within which the parasites rapidly divide, an energetically costly process that may suffer from resource limitation at high densities (Carwardine and Hurd 1997; Hogg and Hurd 1995). Finally, once sporozoite stage parasites have left the oocyst and colonised the salivary glands, mosquito feeding behaviour may be altered either through behavioural manipulation or direct clogging of the salivary glands (Anderson *et al.* 1999; Koella *et al.* 1998), impacting vector survival and parasite transmission (Anderson *et al.* 2000). Additionally, across all stages, parasites are also vulnerable to the vector immune responses and the strength of these responses may depend on both the density of malaria parasites and the introduction of bacteria when migrating ookinete numbers are high (Cirimotich *et al.* 2010; Michel and Kafatos 2005; Rodrigues *et al.* 2010). The impact of these factors on parasite fitness may be additive and will also be influenced by the environmental conditions experienced by the vector (Fellous and Koella 2010; Ferguson and Read 2002b; Lambrechts *et al.* 2006). Although parasite fitness will be determined by both vector survival and the ability of parasites to colonise the salivary glands, parasite productivity is rarely studied and the potential additive effect of these factors is largely ignored (but see Churcher *et al.* 2010; Dawes *et al.* 2009b). Due to the current drive to develop transmission blocking interventions, understanding how the density of malaria parasites impacts processes within the vector is timely and important (Churcher *et al.* 2010).

Here I report the results of a large study using experimentally generated variation in infection intensity, to investigate the consequences of density-dependence and mosquito environmental conditions for parasite transmission success. By using a combination of *in vivo* and *in vitro* experiments I examine parasite development through the life-cycle. I predict that if parasites experience crowding in high density infections their productivity will be reduced. In addition, if high density infections are costly for mosquitoes, they will be less likely to survive for long enough for parasites to complete development.

6.3. Methods

The transmission of malaria parasites through the mosquito, termed sporogony, involves sexually differentiated stages (gametocytes) being taken up in a blood meal where they rapidly differentiate into male and female gametes and mate (Bannister and Mitchell 2003; Ross 1897). Within 18-20 hours post fertilisation, each zygote transforms into a motile ookinete, which traverses the midgut wall, invades the epithelium of the vector and differentiates into an oocyst. The parasites replicate asexually to produce thousands of sporozoites inside each oocyst before rupturing and releasing sporozoites into the haemocoel. Once sporozoites have migrated to the salivary glands they are ready to be injected into new hosts during the next blood feed (Bannister and Mitchell 2003; Beier 1998). For *P. berghei* in *Anopheles stephensi*, sporogony takes around 21 days (Figure 5.1; Sinden *et al.* 2007). I measure mosquito mortality rates and parasite densities at each stage in sporogony. This experimental design enables me to identify which sporogonic stages drive density dependent consequences of infection for both parasite proliferation and harm to the mosquito.

6.3.1. Parasites, hosts and vectors

This study uses two lines of *Plasmodium berghei*, Pb820cl1m1cl1 (RMgm-164; Ponzi *et al.* 2009) and PbMC1-KO (RMgm-153; kind gift from Dr. Shahid Khan, Leiden malaria group), both originating from the 'high gametocyte producing' ANKA strain. The PbMC1-KO line has been deleted for metacaspase 1 and was initially generated to investigate if this gene is essential for apoptosis of ookinetes. However previous studies have revealed no significant cell death phenotype associated with PbMC1 (Le Chat *et al.* 2007). I also find that PbMC1 is not essential for parasite apoptosis, as ookinetes in both the PbMC1 and Pb820 lines display DNA fragmentation, a marker for apoptosis (TUNEL *in situ* cell death detection kit, Fluorescein; Roche, n = 5 infections/line). However, PbMC1 has been found to result in high density infections in mosquitoes (S. Khan and M. Shahid personal communication) which I confirmed in a pilot study (Mean oocyst density per mosquito; PbMC1 = 376 (±48.9); Pb820 = 268 (±34.6); n = 6 cages of 75

mosquitoes/line). For clarity the Pb820cl1m1cl1 line is hereafter referred to as regular density (RD) line and PbMC1-KO as high density (HD) line.

All hosts were 8-12 week old male MF1 mice (in-house supplier, University of Edinburgh). Experimental infections for transmission to mosquitoes were initiated with $5x10^7$ parasitised red blood cells of the RD or HD lines (n=12 infections per line). On day 3 post infection, the day of transmission, thin blood smears were taken and red blood cell densities were estimated using Flow Cytometry (Beckman Coulter Counter; see Ferguson *et al.* 2003). The gametocytemia (proportion of red blood cells infected with gametocytes) and sex ratio (proportion of gametocytes male) was estimated by microscopy, and the density of gametocytes in blood meals was calculated as the red blood cell density multiplied by gametocytemia. Each of the 24 infected mice, plus an additional 8 uninfected control mice (age- and sex-matched) were anaesthetised (1.7 parts Dormitor, 1.3 parts Vetelar in 7 parts PBS given at 4µl per g) and each mouse was exposed to a single experimental cage of mosquitoes for 30 minutes. Any unfed mosquitoes were removed from the cages (< 5 per cage). All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Anopheles stephensi mosquito colonies were maintained under standard insectary conditions of 27±1° C, 70% humidity and 12:12 light:dark cycle at the University of Edinburgh. Larvae were reared in plastic trays at a density of 250/1.5L of distilled water and on days 11-13 after egg hatching, pupae were collected and transferred to large emergence cages (1 cage per day) with *ad libitum* access to 10% glucose solution supplemented with 0.05% paraminobenzoic acid. On day 6-8 post-emergence, female mosquitoes were removed from emergence cages and transferred to 32 1.5L experimental cages, each containing 75-80 individuals. Each experimental cage contained females randomly chosen from each of the 3 emergence cages and mosquitoes were subsequently housed in a 21°C incubator (humidity 50±5%). All mosquitoes were starved for 24hrs before blood meals and each cage was exposed to an infected or uninfected host for 30 minutes to feed. Of the 32 experimental cages, 12 received RD parasites, 12 received HD parasites, and 8 received uninfected blood (control). Hydric stress and nutrient depletion have previously been found to be

important in determining the virulence of malaria infection to mosquitoes (Aboagye-Antwi *et al.* 2010; Ferguson and Read 2002b; Lambrechts *et al.* 2006). Therefore, half of the experimental cages for each parasite line (n=6 per condition) and control feeds (n=4 per condition) were kept in 'standard' conditions with *ad libitum* access to glucose and water and half were kept under 'restricted' conditions where they only had access 50% of the time (following Ferguson and Read 2002b). Thus, in total, 32 experimental cages with 2187 mosquitoes contributed data to the analyses. This number of replicate cages for each line and treatment combination is necessary as mosquitoes sharing a cage are not statistically independent data points.

6.3.2. Generation of different infection intensities

My experiment required that the RD and HD lines generated variation in the intensity of infections in mosquitoes and I validated this by measuring the densities of ookinetes and oocysts. Ookinetes are the most readily identifiable post-fertilisation form but their density inside blood meals is difficult to accurately assay. Therefore, *in vitro* cultures that mimic vector conditions are the standard way to measure parasite fertilisation rates and reproductive success (Janse *et al.* 1985; Ramiro *et al.* 2011; Reece *et al.* 2008). I infected 12 hosts with 1x 10*7 parasitised red blood cells of either the RD of HD lines (using the HD clone, PbMC1521cl$_1$). These hosts were pre-treated with Phenyl hydrazine (PHZ; 120mg/kg) 4 days prior to infection to increase reticulocytaemia which stimulates the production of gametocytes (Gautret *et al.* 1996). One host in the RD group did not develop an infection and was therefore excluded from the study. On day 4 post-infection, 4 replicate cultures per infection were set up from 75µL of infected blood in 5mLs of complete ookinete culture media (RPMI + 10% fetal calf serum, pH 8). Gametocyte densities and sex ratios were also estimated from thin blood smears and flow cytometry. After 18 hours incubation at 21°C cultures were vortexed and 3 x 100µL aliquots were taken from each flask. Mature ookinete density was counted from these aliquots using a haemocytometer and for each culture the density of ookinetes per mL of infected blood was calculated using the mean of the 3 aliquots. To assay oocyst density, 10 mosquitoes were randomly selected and removed from each cage on day 14 post infection. Mosquitoes were anaesthetised with chloroform and dissected to extract their midguts. The

infection status (positive or negative) and the number of oocysts per midgut were recorded. The same number of mosquitoes from control cages were removed and discarded.

6.3.3. Vector mortality and parasite proliferation

Testing my hypotheses required assaying parasite proliferation (the production of sporozoites) and measuring the mortality rate of mosquitoes. On day 21 post-infection, I randomly selected 10 mosquitoes per cage to determine infection status and the density of sporozoites in the salivary glands. Sporozoite presence and density was quantified by homogenising salivary glands in 20µL PBS before counting sporozoites on a haemocytomer. The same number of mosquitoes from control cages were removed and discarded. To estimate vector mortality rates, cages were checked every second day, through sporogony (days 0-21) and until day 50 when less than 2% of all infected and uninfected mosquitoes remained alive. All dead mosquitoes were counted and removed. The mosquitoes sampled for counts of oocysts or sporozoites and the few remaining alive on day 50 (< 6 per cage) were counted as censored data points in the mortality analysis (Crawley 2007). One pot (RD line under restricted conditions) was removed from survival analysis due to missing individuals.

6.3.4. Statistical analyses

All analyses were performed using R version 2.12.1 (The R foundation for statistical computing; http://www.R-project.org). Survival analysis was performed using Cox proportional hazard mixed effect models with experimental cage fitted as a random effect to overcome pseudoreplication problems of sampling multiple mosquitoes from each cage (Terry Therneau (2009) coxme: Mixed Effects Cox Models. R package version 2.0.). Infection rates (# mosquitoes infected/# dissected per pot at each stage) and gametocyte sex ratios (proportion male) were analysed using generalized linear models with binomial error structures. Gametocyte density of the two lines was compared with a general linear model. The differences in ookinete, oocyst and sporozoite (log transformed for normality) densities between the two lines were compared using linear mixed effect models with mouse (ookinete data) or cage

(for oocyst and sporozoite data) fitted as random effects. To examine the effect of oocyst density on mosquito survival and parasite productivity, I compared the mean oocyst and sporozoite densities per cage (log transformed for normality) and the proportion of mosquitoes surviving to day 21 post infection (arcsine square root transformed) using general linear models. With the exception of the survival analysis, where the model output is reported, I followed model simplification by sequentially dropping the least significant term and comparing the change in deviance with and without the term to χ^2 distributions until the minimal adequate model was reached. Degrees of freedom correspond to the difference in the number of terms in the model.

6.4. Results

6.4.1. Generation of different infection intensities

Due to the practical difficulties in accurately estimating ookinete densities from dissected midguts I examined the phenotypes of the two parasite lines *in vitro*. The HD line generated more than four times the number of ookinetes than the RD line (Ookinetes per ml of infected blood: RD = 23800 (± 2466 S.E.); HD = 104167 (± 7877 S.E.); $\chi^2_1 = 16.045$, $p < 0.0001$; Figure 6.1a). This difference resulted both from a slightly higher gametocyte densities following PHZ treatment (mean gametocytes per ml of blood: RD = 1.10×10^{10} ($\pm 8.57 \times 10^8$ S.E.); HD = 1.86×10^{10} ($\pm 1.14 \times 10^9$ S.E.); $\chi^2_1 = 5.81$, $p = 0.016$) and an increased fertilisation rate in the HD line (% females fertilised: RD = 3.5×10^{-3} ($\pm 4.5 \times 10^{-4}$ S.E.); HD = 6.6×10^{-3} ($\pm 4.4 \times 10^{-4}$ S.E.); $\chi^2_1 = 6.436$, $p = 0.0112$).

The proportion of mosquitoes infected with oocysts was high (> 80%) and was not affected by either the conditions the mosquitoes experienced (standard vs. restricted; $\chi^2_1=0.074$, $p=0.39$) or parasite line ($\chi^2_1=1.54$, $p=0.22$). I then compared the densities of oocysts generated by the two lines. The conditions mosquitoes were kept under did not affect oocyst density ($\chi^2_1 = 0.0002$, $p = 0.99$). As expected, infection with the HD line resulted in more than twice as many oocysts per infected mosquito (RD = 230 (± 17 S.E.); HD = 519 (± 38 S.E); $\chi^2_1 = 27.19$, $p < 0.0001$; Figure 6.1b). This difference cannot be explained by line differences in either the sex ratio or density of

gametocytes in the infections at the time of transmission (proportion male χ^2_1=0.158, p=0.69; Gametocytes per ml of blood χ^2_1=0.499, p=0.50).

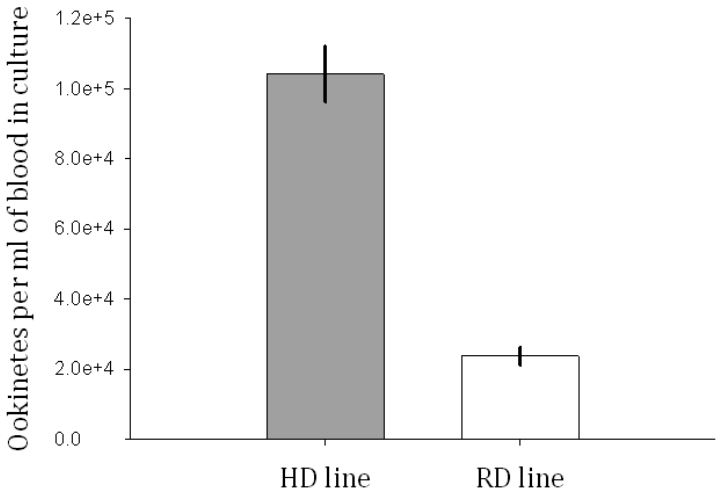

Figure 6.1a: Generation of different infection densities: Ookinetes. Number of ookinetes at 18 hours resulting from culture per 1mL of infected blood based on between 5 and 6 replicate infections and between 20 and 24 cultures per line. High density line (filled bar) and the regular density line (open bar). Error bars show the standard error of the mean.

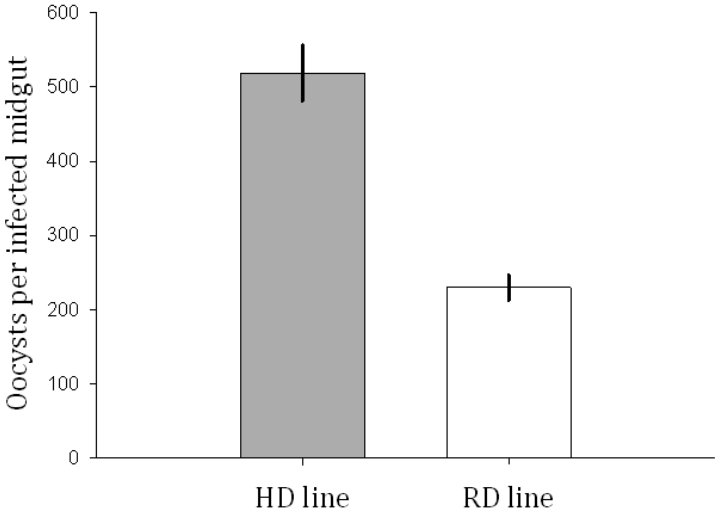

Figure 6.1b: Generation of different infection densities: Oocysts. Number of oocysts per midgut of infected mosquitoes based on 12 pots per line with 10 mosquitoes per pot. High density line (filled bar) and the regular density line (open bar). Error bars show the standard error of the mean.

6.4.2. Parasite proliferation

Having confirmed that infection with the HD line resulted in significantly higher numbers of ookinetes and oocysts than infection with the RD line, I then examined the consequences for parasite productivity, measured by the density of sporozoites successfully reaching the salivary glands. For *P. berghei* parasites infecting *An. Stephenensi,* sporozoites reach the salivary glands and can therefore be transmitted by 21 days after an infected blood-meal; they are not seen to increase in density after this point (Dawes *et al.* 2009b). The proportion of mosquitoes with detectable sporozoites was not influenced by the conditions mosquitoes experienced (standard vs. restricted), and although the proportion of mosquitoes with sporozoites was slightly lower for the HD line, this was not significant (Table 6.1).

Analysis of the entire data set did not demonstrate an effect of parasite line, the conditions mosquitoes experienced or an interaction between line and condition on sporozoite density in the salivary glands. However, as both the effect of parasite line and the interaction with condition were borderline, I examined the effect of line in each of the two conditions separately. For mosquitoes in standard conditions, the HD line produced significantly fewer sporozoites per mosquito than the RD line. Under restricted conditions, there was no significant effect of parasite line and sporozoite densities were intermediate between the two lines in standard conditions. As these results suggested a negative effect of high oocyst numbers on sporozoite production, I examined this in more detail by comparing the mean densities of oocysts and sporozoites for each cage. Across all treatment groups mean sporozoite density was negatively correlated with mean oocyst density, and there was no additional effect of line, condition or the interaction between line and condition (Table 6.1., Figure 6.2b). This supported my findings that high density infections are detrimental to parasite productivity.

Line effect on presence and density of salivary gland sporozoites (day 21)			
		Mean (±S.E)	
Sporozoites per mosquito (log)	All data		Condition χ^2_1=0.039, p=0.84 Parasite line χ^2_1=3.68, p=0.055 Line*condition χ^2_1=3.28, p=0.06
	Restricted conditions - Regular density - High density	7.78 (±0.31) 7.49 (±0.46)	Parasite line χ^2_1=0.034, p=0.85
	Standard conditions - Regular density - High density	8.34 (±0.41) 6.80 (±0.36)	**Parasite Line χ^2_1=5.71,p=0.017**
Proportion of Mosquitoes infected with sporozoites		39% (±3%)	Parasite line χ^2_1=3.46, p=0.063 Condition χ^2_1=0.004, p=0.95

Relationship between mean oocyst density and mean sporozoite density per cage			
		Intercept	slope
Mean sporozoite density (log)	**Mean oocyst density χ^2_1= 18.29, p=0.0004** Parasite line χ^2_1=0.023, p=0.88 Condition χ^2_1=1.94, p=0.18 Oocyst density*parasite line χ^2_1=0.25, p=0.62	11.22	- 0.099

Table 6.1: Density effects on parasite proliferation. Statistically significant results highlighted in bold.

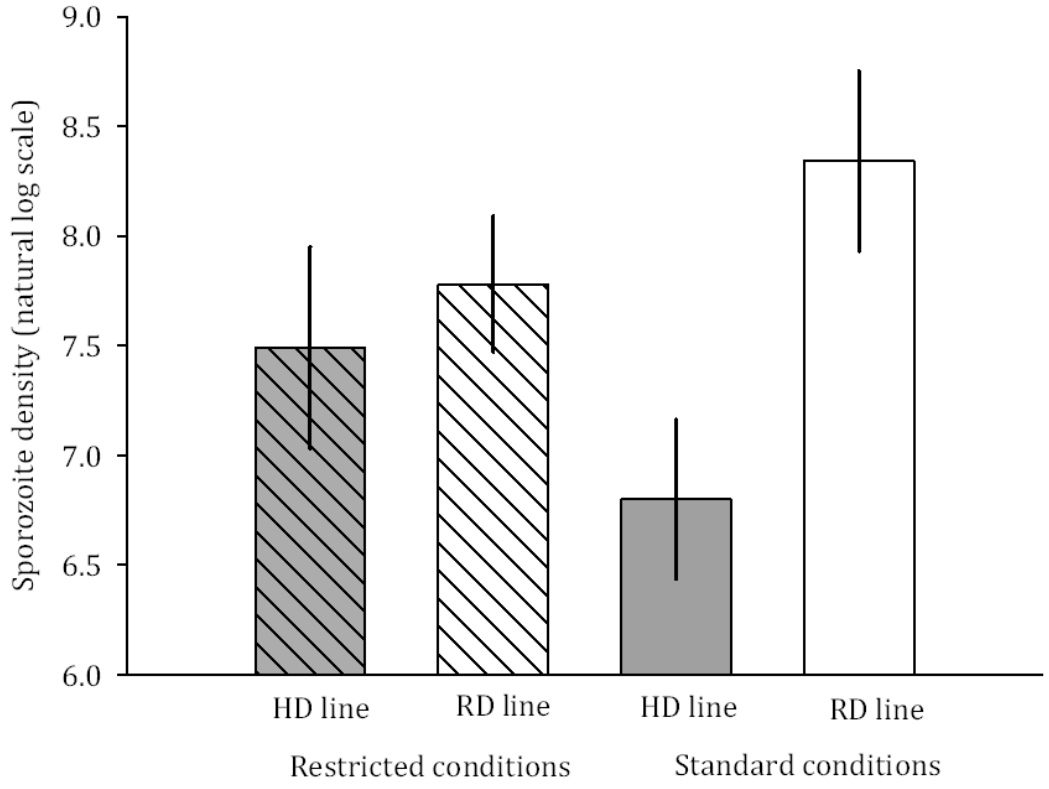

Figure 6.2a: Density effects on parasite proliferation: sporozoite density by group. The mean sporozoite density per mosquito on day 21 post infection for the regular density (white) and high density (grey) lines under standard (solid bars) and restricted (hashed bars) conditions. Means are based on 6 replicate cages with 10 mosquitoes dissected per cage. Bars show the standard error of the mean and data is on a log scale to fit a normal distribution.

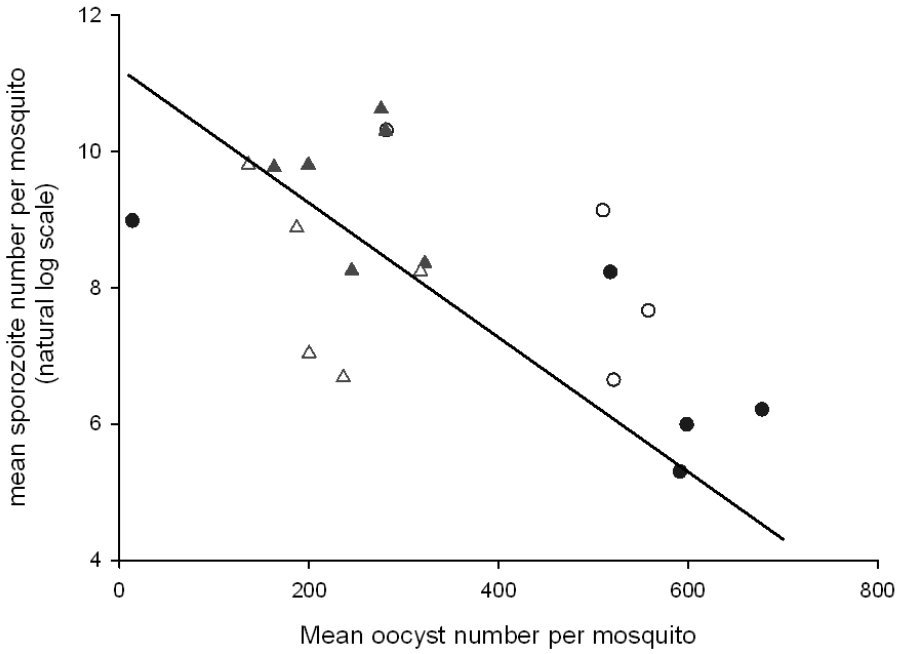

Figure 6.2b: Density effects on parasite proliferation: Sporozoite density by mean oocyst density per cage Relationship between number of oocysts and density of sporozoites in the salivary glands at day 21 in infections with the regular density line (triangles) or the high density line (circles) under either standard conditions (solid symbols) or restricted conditions (open symbols). The line shows the predicted relationship from the minimal model (Table 6.1.). Each point represents the mean value for one replicate cage of mosquitoes with 10 mosquitoes dissected per cage at each stage. Data are plotted on a natural log scale.

6.4.3. Vector survival

6.4.3.1. Survival during sporogeny

Under standard conditions, mosquitoes infected with the HD line were significantly less likely to survive to day 21 (when sporozoites reach the salivary glands) than both control uninfected mosquitoes and mosquitoes infected with the RD line. However, the survival of mosquitoes infected with the RD line and control uninfected mosquitoes did not significantly differ (Table 6.2.; Figure 6.3a). This suggests that infections with high oocyst densities are costly, therefore I compared the mean oocyst densities per cage to the proportion of mosquitoes surviving to day 21 and found a strong negative relationship for RD infections in standard and restricted conditions, but not for HD infections in either condition (Table 6.2; Figure 6.4.). Keeping mosquitoes under restricted conditions significantly reduced survival over the first 21 days of infection (Coxme: dead=880, total=2187, Z=4.91, $p<0.00001$; Figure 6.3b), but there was no additional significant influence of whether mosquitoes were infected with RD, HD or uninfected controls.

6.4.3.2. Longer-term survival

Most studies examining the effect of malaria infection on mosquito survival only follow the vectors to the point where transmission can occur, however, as sporozoites remain in the salivary glands after this point (Dawes *et al.* 2009b), longer-term vector survival can influence parasite transmission. Survival to day 50 under standard conditions was significantly lower in both of the infected treatments than the control but there was no significant difference between infection with the RD and HD lines (Table 6.2; Figure 6.3a inset). Keeping mosquitoes under restricted conditions significantly reduced longer term survival (Coxme: dead=1451, total=2187, Z=5.70, $p<0.00001$; Figure 6.3b inset). As with survival during sporogony, under restricted conditions there was no significant influence of whether mosquitoes were infected with RD, HD or were uninfected controls.

Standard conditions			
		n dead, n total	Coxme analysis
Survival to day 21	**HD vs. control**	**218, 710**	**Z=3.26, p=0.001**
	HD vs. RD	**276, 841**	**Z=2.08, p=0.037**
	RD vs. control	176, 701	Z=1.46, p=0.15
Survival to day 50	**HD vs. control**	**450, 710**	**Z=4.09, p<0.0001**
	HD vs. RD	539, 841	Z=1.77, p=0.077
	RD vs. control	**437, 701**	**Z=2.53, p=0.011**
Mean oocyst density and proportion of mosquitoes surviving to day 21			
	Intercept	slope	
RD	**1.35**	**-0.0014**	**χ^2_1=22.13, p<0.005**
HD	NS	NS	χ^2_1=1.00, p=0.34
Restricted conditions			
		n dead, n total	Coxme analysis
Survival to day 21	HD vs. control	359, 710	Z=0.05, p=0.96
	HD vs. RD	395, 776	Z=0.17, p=0.86
	RD vs. control	332, 636	Z=0.11, p=0.91
Survival to day 50	HD vs. control	498, 710	Z=0.48, p=0.63
	HD vs. RD	539, 776	Z=0.41, p=0.68
	RD vs. control	439, 636	Z=0.67, p=0.5
Mean oocyst density and proportion of mosquitoes surviving to day 21			
	Intercept	slope	
RD	**1.01**	**-0.0014**	**χ^2_1=22.13, p<0.005**
HD	NS	NS	χ^2_1=1.00, p=0.34

Table 6.2: Parasite oocyst stage density and mosquito lifespan. High density (HD) and regular density (RD). Significant effects are highlighted in bold.

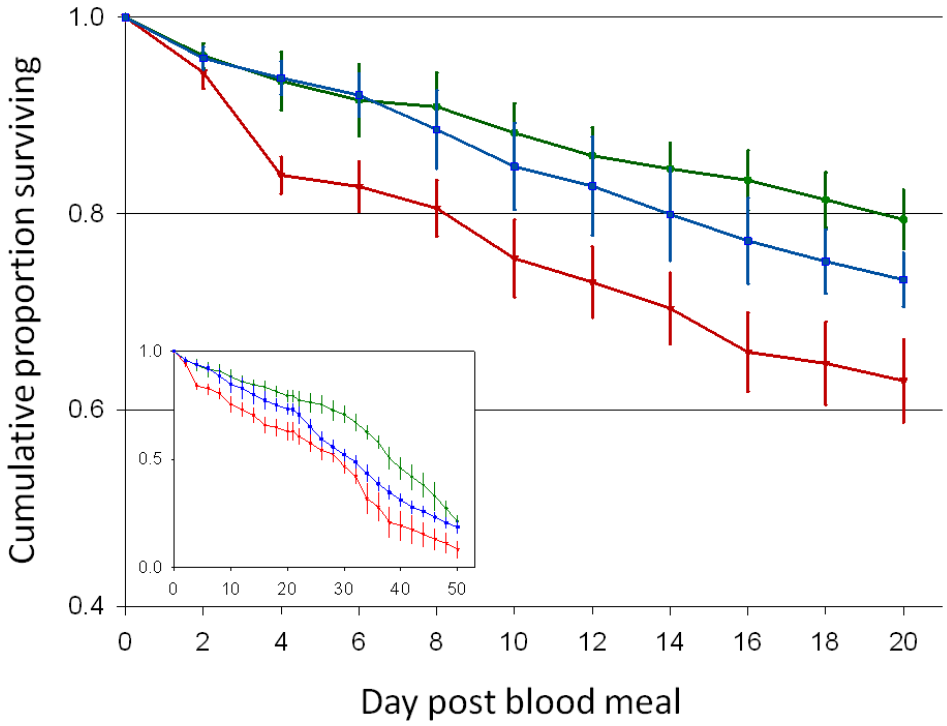

Figure 6.3a: Density effects on vector survival: standard conditions. Cumulative proportion of mosquitoes surviving until day 21 (when the sporozoites reach the salivary glands) after receiving a control uninfected blood meal (green), infection with the regular density line (blue) or the high density line (red) under standard glucose and water conditions. Inset shows the full 50 days for which mortality was recorded. Each point represents the mean survival for between 4 and 6 cages and the error bars show the standard error of the mean.

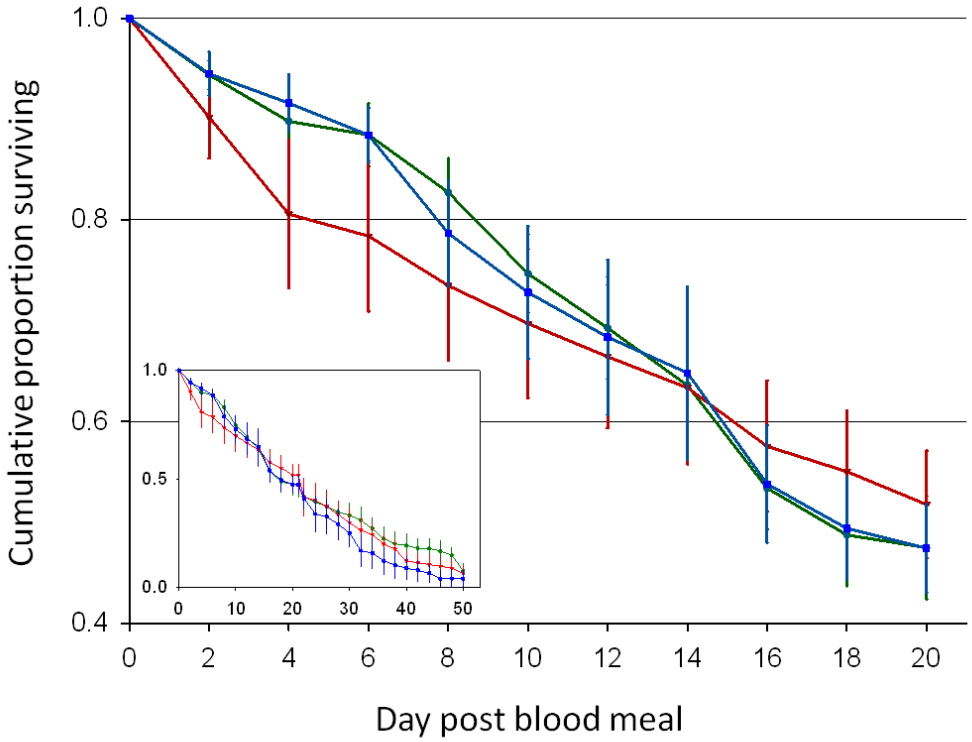

Figure 6.3b: Density effects on vector survival: restricted conditions. Cumulative proportion of mosquitoes surviving until day 21 (when the sporozoites reach the salivary glands) after receiving a control uninfected blood meal (green), infection with the regular density line (blue) or the high density line (red) under restricted glucose and water conditions. Inset shows the full 50 days for which mortality was recorded. Each point represents the mean survival for between 4 and 6 cages and the error bars show the standard error of the mean.

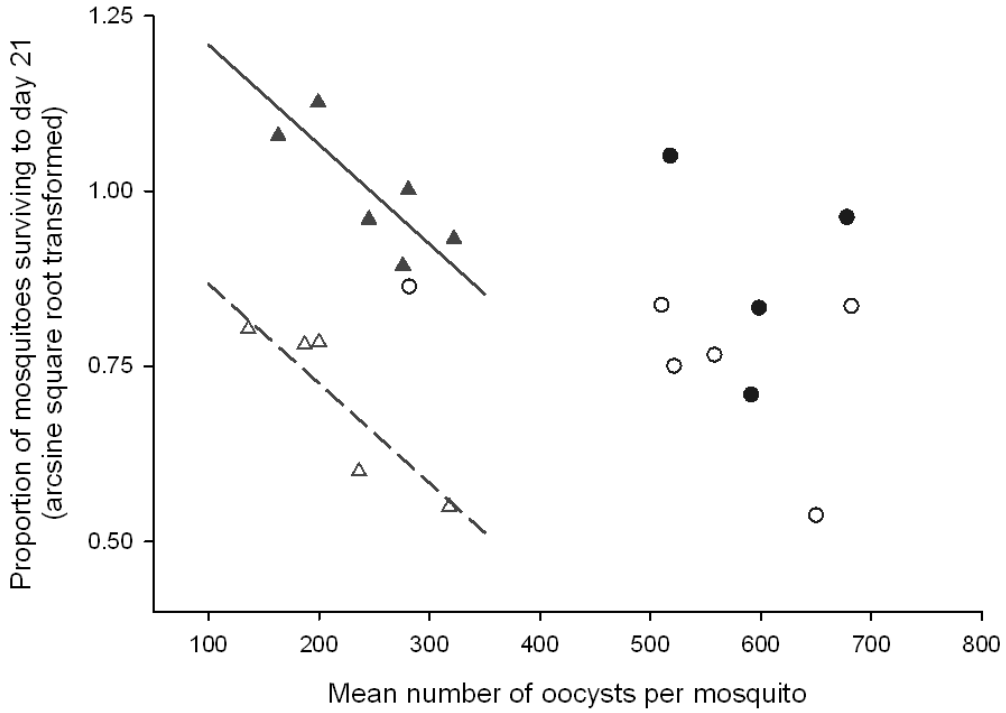

Figure 6.4: Oocyst density and mosquito lifespan. The relationship between oocyst density and proportion of mosquitoes surviving to day 21 post infection (arcsine square root transformed) for infections with the regular density line (triangles) or the high density line (circles), under either standard conditions (solid symbols) or restricted conditions (open symbols).

6.5. Discussion

My data demonstrate that density-dependent processes can be costly to parasites in two ways. Firstly, infections with high densities of ookinete and oocyst stage parasites result in decreased productivity, with lower numbers of transmissible sporozoite stage parasites reaching the salivary glands. Secondly, vectors with high density infections are more likely to die before the completion of sporogony. Furthermore, the relative cost of high density infection varies depending on environmental conditions. I successfully generated infections in mosquitoes with elevated densities, which had on average more than double the oocyst burden per midgut of the regular density line. When vectors were kept under standard conditions, parasites in high density infections experienced an 80% reduction in the number of salivary gland sporozoites compared to parasites in regular density infections. While it is not possible to entirely rule out additional confounding differences between the two lines, the impact of high oocyst densities on sporozoite productivity was strongly supported by a negative correlation between the mean oocyst and mean sporozoite density per cage across all treatment groups. The magnitude of the effects reported may be conservative as even the regular density line produces higher oocyst burdens than have been observed in natural infections with human malaria (Tripet *et al.* 2008). However, if mosquitoes with heavy oocyst burdens are less likely to survive, they will be harder to find in the wild.

High densities of ookinetes and oocysts could limit the ability of parasites to produce sporozoites and successfully colonise the salivary glands through competition for space and resources limiting the number of sporozoites produced per oocyst. Additionally, stronger vector immune responses could also limit salivary gland sporozoites either through melonisation of developing oocysts or by killing sporozoites on their way to the salivary glands (Cirimotich *et al.* 2010; Dimopoulos *et al.* 2001; Michel and Kafatos 2005). It is likely that both resource and immune mediated competition will contribute to the reduced productivity in high density infections and while it is not possible to determine the relative contribution of these factors in my study, a role for resource limitation is supported by my data: sporozoite numbers are lower under restricted conditions. Future experiments could shed further

light on this issue by utilising the growing tool set for quantifying insect immune responses (Cirimotich *et al.* 2010; Michel and Kafatos 2005; Tripet *et al.* 2008).

The number of sporozoites injected in an infective bite is thought to be related to the chance of parasites establishing an infection (Kebaier *et al.* 2009), therefore my sporozoite data alone suggest that high density infections will suffer reduced transmission rates. Additionally, my mortality data show that high oocyst burdens can also reduce the proportion of vectors surviving until sporozoites reach the salivary glands. While the cost of high oocyst densities was greatest early in the infection, more generally, my data show that mosquitoes infected with both regular and high density infections experienced increased overall mortality compared to uninfected controls. The relationship between oocyst density and mortality supports my finding that the difference between lines was greatest early in the infection and suggests that parasite-inflicted damage to mosquitoes occurs either during the ookinete passage through the midgut and/or during oocyst development. This finding is in keeping with previous studies showing that mosquitoes suffer an energetic cost of high density infections which is most likely to be paid while parasites are rapidly replicating in oocysts (Carwardine and Hurd 1997; Hogg and Hurd 1995). Furthermore, ookinetes traversing the midgut lead to death of epithelium cells, and although the epithelium repairs and seals (Han and Barillas-Mury 2002), high densities of ookinetes may lead to permanent damage and risk of secondary bacterial infections (Dimopoulos *et al.* 2002; Meister *et al.* 2009). More broadly, my results support the controversial hypothesis that malaria is bad for mosquitoes (Ferguson and Read 2002a) and suggest that where costs of infection have not been detected, this could be due to short monitoring periods (e.g. Robert *et al.* 1990).

Under natural settings, mosquitoes are likely to experience varying levels of hydric and nutrient stress depending on habitat and season, and this can impact parasite-vector interactions (Tripet *et al.* 2008). As expected, restricting sugar and water increased vector mortality but also had significant fitness costs for malaria parasites, reducing both sporozoite density and vector survival during sporogony. Whilst the effect of infection density on sporozoite production and vector survival are not as clear under restricted conditions, the patterns are qualitatively similar to standard (*ad*

libitum) conditions. In particular, vector survival drops sharply in the first few days of high density infections. While, initially, it seems surprising that the effect of infection density was greatest in standard conditions, this could be due to the combined effect of nutrient and hydric stress on mosquito mortality masking the costs of infection in restricted conditions. The effect of vector conditions on parasite transmission may not be straightforward, as nutrient limitation will influence both the resources available to the developing parasites and the resources available for the vector to mount an immune response against the parasite (Schmid-Hempel 2005; Tripet *et al.* 2008). My results demonstrate the importance of environmental variation in determining parasite dynamics and interactions with the vector. However, future studies examining the shape of these relationships over a range of different quality environments and parasite densities are needed.

Previous research has suggested that the relationship between ookinete density and salivary gland sporozoites is saturating, with more ookinetes no longer increasing sporozoite density after a threshold level (Churcher *et al.* 2010; Sinden *et al.* 2007). My data crucially adds that this relationship is negative at high densities. This has important implications for transmission blocking interventions as their success is generally judged in terms of a reduction in oocyst number (Chowdhury *et al.* 2009; Miura *et al.* 2007). My results suggest a reduction oocyst density may not result in reduced transmission and may actually have the opposite effect though increased vector survival and sporozoite production. These effects are likely to be particularly important as the development time required for malaria parasites to be transmissible from the salivary glands (~3 weeks; Sinden *et al.* 2007a) is long compared to the average life expectancy of mosquitoes in the wild, which is estimated as low as 1-2 weeks for adult females of the *Anopheles* species (Gwadz and Collins 1996). Furthermore, the impact of the relationships described here may be amplified in the wild as oocyst densities in infected mosquitoes are highly variable (Medley *et al.* 1993) and parasite aggregation will generally increase the impact of density-dependent processes on the transmission of parasites (Anderson and May 1978; Churcher *et al.* 2005). Therefore, slight variations in the mortality rates of mosquitoes or on their ability to reach the salivary glands could have a significant effect on parasite transmission (Macdonald 1957). Gaining a better quantitative

knowledge of the relationships between oocyst burdens, sporozoite production, and vector survival will be crucial for predicting the effects on transmission of both natural variation and deliberate changes in ookinete/oocyst burdens, for example, as a result of interventions.

The relationship between parasite density and transmission may be further complicated by the mounting evidence that malaria ookinetes undergo apoptosis (Al-Olayan *et al.* 2002; Hurd and Carter 2004; Pollitt *et al.* 2010) which is proposed to be an altruistic behaviour (Al-Olayan *et al.* 2002; Duszenko *et al.* 2006; Welburn *et al.* 2006). The occurrence of, and putative explanation for, apoptosis in protozoan parasites is controversial, but evolutionary theory can help to make clear predictions for when and why altruistic behaviours will be observed (Foster 2005; Hamilton 1963; Hamilton 1964; West *et al.* 2006). For malaria parasites to evolve altruistic apoptosis, a key requirement is that the surviving parasites must benefit from density regulation - high density infections incur fitness costs for parasites by reducing transmission, through premature vector mortality or because crowding reduces sporozoite production. My data demonstrate that both of these costs occur and support the hypothesis that natural selection would favour parasite genotypes that actively regulate their density. The high density line, in which metacaspase 1 is disrupted, was initially created to investigate if this gene is essential for apoptosis of ookinetes. Whilst these experiments were not designed to investigate the function of metacaspase 1 a marker for apoptosis was recorded in ookinetes of both the lines. This is in keeping with findings of previous studies suggesting metacaspase 1 is not essential for apoptosis (Le Chat *et al.* 2007). However, while an inability to undergo apoptosis does not explain elevated ookinete densities in the high density line, metacaspase may be involved in determining an appropriate apoptosis response to parasite density. Data suggest that the gametocyte investment and sex ratio decisions parasites make while in the vertebrate host are density dependent (Pollitt *et al.* 2011b; Reece *et al.* 2008), but whether they can employ such sophisticated strategies in the vector remains unknown.

In conclusion, my data demonstrate that there are significant fitness costs to parasites in high density infections, including reduced numbers of transmission stages and

high vector mortality. Understanding the impact of these factors on the transmission of disease and predicting the impact of transmission blocking intervention strategies will require more complete knowledge of the density-dependent processes taking place within the vector (Churcher *et al.* 2010). As for all ecological interactions, the shape of density-dependent relationships is complex and influenced by many factors, including nutrient availability and the presence of competitors (Krebs and Davies 1997). Clearly, this is an area where the integration of detailed, well-replicated experimental data with mathematical modelling can help to make clear, testable predictions to explain observed patterns.

CHAPTER 7

GENERAL DISCUSSION

The aim of this thesis was to use evolutionary ecology to gain insight into transmission strategies of protozoan parasites both within the host and within the vector. Specifically, in this thesis, I have: i) demonstrated that, in accordance with theory, malaria parasites detect and respond to the presence of competitors by altering reproductive strategies to maximise within-host survival, and that strategies are fine tuned in response to variation in within-host environment; ii) applied findings from the evolutionary ecology of malaria parasites to African trypanosomes, showing how trypanosomes can provide new opportunities for studying parasite transmission strategies; iii) demonstrated how the complexity of within-host environments pose specific challenges for statistical investigation and verified methods for robust analysis; iv) Investigated the occurrence of, and the evolutionary reasons for, apoptosis in protozoan parasites; and v) shown that density-dependent processes can result in diverse fitness costs to malaria parasites in the vector. A detailed discussion of each of these findings with respect to the relevant literature is provided at the end of each chapter. Rather than repeat these sections, I will now briefly summarise my findings and how they contribute to the understanding of protozoan transmission strategies. Furthermore, I highlight areas where there are still significant gaps in understanding and suggest avenues for future research.

7.1. Transmission strategies in complex within-host environments

Malaria parasites and trypanosomes cause some of the most serious infectious diseases of humans and livestock. The life-cycles of these parasites involve the production of both asexually replicating stages which maintain the infection within the host and specialised transmission stages which reproduce sexually when taken up by an insect vector to produce stages responsible for between-host transmission. Parasites therefore face a trade-off between within-host replication and between-host transmission that is analogous to the survival-reproduction trade-off faced by all sexually reproducing organisms. The resolution of this trade-off is predicted to have substantial impacts on patterns of transmission and on the harm caused to hosts. Therefore, understanding how environmental factors shape this trade-off is important for disease epidemiology. More generally, it is very difficult to measure reproductive effort in more traditionally studied metazoan taxa, but as it is relatively straightforward to measure investment into reproduction in malaria parasites they provide a novel system for testing life-history theory.

For malaria parasites, there is substantial variation in the densities of transmission stage parasites (gametocytes) observed both within and between infections (Drakeley *et al.* 2006; Stepniewska *et al.* 2008). While this will be explained in part by extrinsic factors (i.e. immune killing), experimental studies show that gametocyte density is also under parasite control - malaria parasites alter their relative investment into transmission in response to characteristics of the within-host environment including anti-parasitic drugs, resource availability and competition (Bousema *et al.* 2008; Buckling *et al.* 1999a; Buckling *et al.* 1999b; Pollitt *et al.* 2011b; Reece *et al.* 2010). I demonstrate that there is genetic variation in the patterns of investment in transmission for *P. chabaudi* parasites and that across genotypes investment is decreased in response to competition. Furthermore, this response can be partly explained by availability of red blood cell resources. These findings fit with theoretical predictions for malaria parasites - in mixed genotype infections parasites should switch investment away from transmission and into within-host replication in order to gain a greater share of exploitable resources and ensure within-host survival (Mideo and Day 2008).

More generally my findings fit with predictions for reproductive restraint – current reproductive effort will be constrained by the need to invest in survival (Fischer *et al.* 2009; McNamara *et al.* 2009). Theory predicts that as conditions deteriorate investment in reproduction will decrease due to the need to invest in survival; however, eventually survival becomes sufficiently unlikely that terminally investing in reproduction is the best strategy (Charlesworth and Leon 1976; Fischer *et al.* 2009; Williams 1966). Results of various studies of malaria parasite investment in gametocytes seem to fit this framework (reviewed in chapter 3), however the exact shape of this relationship is unknown and importantly so is the point at which parasite strategies switch from a strategy of reproductive restraint to terminal investment. Quantifying this relationship is challenging as parasite responses will depend on their ecology - parasite genotypes vary in their relative competitive ability, their preference for particular age cohorts of red blood cells and host immune responses may be more or less cross reactive. For example, while reproductive restraint in response to competition may be the optimal strategy for one genotype, for a poor competitor, a genetically diverse infection may constitute a particularly bad environment, and so terminal investment will be the best strategy.

Understanding how patterns of investment in transmission are shaped by different characteristics of the within-host environment will be complex and involve measuring patterns of investment over time and across a range of conditions. For laboratory systems measuring parasite strategies over the course of the infection is relatively straightforward, as long as the right statistical controls are in place, but natural infections pose more of a challenge. Due to logistical and ethical constraints, data on human malaria infections will generally consist of single snapshots in time (Färnert 2008). Despite this, it may be possible to detect large scale patterns in data across populations. For example, if parasites invest less in transmission in mixed infections then the prediction would be for lower relative gametocyte densities in regions where mixed infections are common. This pattern is seen in existing data, but could be due to immunity to gametocytes developing more quickly than to asexuals in endemic regions (Drakeley *et al.* 2006). Now that the tools are available to measure genetic diversity in natural infections it should be possible to test these hypotheses and control for potentially confounding factors like immune responses.

Another key outstanding question is whether malaria parasites alter their transmission strategies as a general response to stress or whether the level of response will vary depending on the source of the stress. The findings presented in this thesis add to mounting evidence that malaria parasites plastically alter reproduction strategies in response to the presence of con-specific competitors (Pollitt *et al.* 2011b; Reece *et al.* 2008) - does this indicate that they can discriminate kin or that mixed infections result in changes indirectly through altering the within-host environment? Distinguishing the relative importance of direct and indirect responses to competition is challenging due co-variation in the characteristics of within-host environments (e.g. the presence of competitors will also influence resource availability and immune responses). However, if the mechanisms by which malaria parasites detect their environment can be determined it will not only provide information on whether these mechanisms are the same across different forms of stress, but also potentially allow parasites to be 'tricked' into doing the wrong thing. If parasites can be forced into different strategies then it will be possible to quantify the fitness costs and benefits of behaviours, and therefore determine whether the originally observed strategies are optimal. In other words, it would be possible to definitively answer questions like: does reproductive restraint increase competitive ability and future transmission? Furthermore, by altering parasite strategies it may be possible to beneficially modify traits underlying disease transmission or virulence to develop novel control programs.

The evolutionary ecology of malaria parasite life-history strategies is relatively well studied (Mackinnon and Marsh 2010; Mideo 2009; Paul *et al.* 2003; Read and Taylor 2001; Reece *et al.* 2009; West *et al.* 2006). In contrast, research on trypanosomes has remained far more focused on molecular cell biology and immunology. While this means that for trypanosomes there is a relative dearth of information on basic parasite life-histories there is a wealth of tractable tools for genetic analysis as well as detailed knowledge of parasite molecular biology (Dean *et al.* 2009; MacGregor and Matthews 2010). There are clear parallels between the life-cycles of these protozoan parasites with both facing a trade off between within-host replication and between-host transmission, therefore I suggest that lessons from one can usefully be applied to the other. Through the attempts to identify the chemical triggering

production of transmission stages (stumpy induction factor) in trypanosomes it may be possible to get closer to understanding mechanisms of kin discrimination and detection of environmental variation of both parasite species (Pollitt *et al.* 2011a).

7.2. Between-host transmission

For vector-borne parasites, patterns of disease transmission will depend not only on the production of transmission stages within the host but also on density dependent processes within the vector. When gametocytes are taken up in a blood meal they differentiate into gametes and mate to form motile ookinete stages. Ookinetes traverse the midgut wall before forming oocysts, where the parasites replicate, producing thousands of sporozoite transmission stages. Quantifying the relationship between the number of ookinetes crossing the midgut wall to form oocysts and the potential for transmission of parasites to a new vertebrate host is central to both the development of transmission blocking interventions (TBIs) and explaining why ookinetes have been observed to undergo apoptosis. However, the predicted effect of density in these situations oppose – a reduction in oocyst numbers due to TBIs is assumed to reduce transmission, but a reduction in oocyst numbers due to ookinete apoptosis is assumed to increase transmission.

Previous research has suggested that the relationship between ookinete density and salivary gland sporozoites is saturating; after reaching a threshold, more ookinetes do not alter sporozoite density (Churcher *et al.* 2010; Sinden *et al.* 2007). However, my data demonstrates that this relationship is negative at high densities - high oocyst numbers lead to reduced sporozoite densities in the salivary glands. As the number of sporozoites injected in an infective bite is thought to be related to the chance of parasites establishing an infection (Kebaier *et al.* 2009), this effect alone is predicted to result in reduced transmission from high density infections. In addition, I show that high oocyst burdens can increase vector mortality, significantly reducing the proportion of vectors surviving until completion of parasite sporogony. The combined effect of reduced proliferation and increased vector mortality suggest that parasites pay a significant cost of reduced transmission in high density infections. However, as for parasite interactions within the host, parasite dynamics within the

vector are complex and patterns depend on the environmental conditions experienced by the vector.

Under natural settings, mosquitoes are likely to experience varying levels of hydric and nutrient stress depending on habitat and season, and this can impact parasite-vector interactions (Tripet *et al.* 2008). In agreement with previous studies (Ferguson and Read 2002b), my data demonstrate that environmental conditions shape the impact of malaria infection on mosquito survival and suggest that vector environments influence the effect parasite density has on proliferation. The influence of the environment on parasite dynamics within the vector is expected to be complex as nutrient availability will determine both the resources available to developing parasites and the resources available for the vector to mount an immune response against the parasite (Schmid-Hempel 2005; Tripet *et al.* 2008). Furthermore, interactions may be shaped by vector and parasite genotypes (GxG) and the presence or absence of co-infecting parasites (Thompson 2005). For example, mosquito immune responses mounted against bacteria are also active against malaria parasites and exposure to bacteria will be environmentally determined (Cirimotich *et al.* 2010; Michel and Kafatos 2005; Rodrigues *et al.* 2010). Understanding the effects of parasite density on sporogony and transmission will be complex, but recent years have seen impressive advances in the tools available to examine insect immune responses and a concurrent increase in the understanding of parasite-vector interactions (Cirimotich *et al.* 2010; Matthews 2011; Michel and Kafatos 2005; Tripet *et al.* 2008). Combining these new tools and understanding with studies on vector ecology will allow the interactions between parasite density, vector environment and their interactions to be better quantified. This will be particularly important for predicting the likely impact of transmission blocking interventions (Churcher *et al.* 2010).

The observed relationship between parasite density and both vector mortality and parasite proliferation indicates there would be a fitness benefit to regulating infection intensity in the mosquito. This supports current thinking about why apoptosis has been observed in ookinete stages of malaria parasites (Al-Olayan *et al.* 2002; Hurd *et al.* 2006; Pollitt *et al.* 2010). However, while this suggests that density regulation

mechanisms could be selected to evolve, determining whether apoptosis is an altruistic trait will involve getting a better understanding of the conditions under which it occurs. If parasites undergo apoptosis to increase transmission of remaining parasites, then rates of apoptosis are predicted to be shaped by both parasite density and relatedness. However, both these predictions remain to be tested and conducting these conceptually simple experiments is limited by the availability of high throughput assays using markers of apoptosis which are relevant to the parasite ecology. In chapter 5 I review these issues and suggest that collaboration between ecologists and cell biologists can help through designing assays and experiments capable of testing the key predictions for when and why apoptosis will occur. However, once these issues are resolved malaria parasites may provide a useful system for testing social evolution theory on the evolution of altruism as the scale of interaction is known (the mosquito) and the fitness outcomes (transmission) are relatively straight forward to determine.

7.3. Concluding remarks

The research presented in this thesis demonstrates the importance of environmental factors in shaping parasite transmission within the host and within the vector, and highlights the complexity in these relationships. This variation poses substantial challenges for predicting patterns of parasite transmission but also provides exciting opportunities for testing the explanatory power of evolutionary biology.

Within-host evolutionary ecology and life-history strategies are comparatively well studied in the rodent malaria system and consequently there are a large number of tools available for the study of parasite transmission strategies, including: genetic tools for tracking genotypes, culture techniques for transmission stages, and predictions from theoretical models (e.g. Antia *et al.* 2008; Babiker *et al.* 2008; Janse *et al.* 1985; Wargo *et al.* 2006). While this has resulted in a relative wealth of data on parasite transmission strategies, there are still significant gaps in our knowledge. For example, the mechanism by which parasites detect characteristics of their within-host environment and the relative contributions different within-host characteristics in

shaping variation in transmission investment within infections, and within and between genotypes, remain unknown. An even greater challenge for understanding patterns of transmission is linking within-host and between-host processes (Mideo *et al.* 2008a). This is limited by a relatively poor understanding of within-vector parasite strategies, interactions, dynamics and the resultant implications for patterns of parasite transmission.

With so many outstanding questions in this relatively well studied system the prospects for applying research to other parasite systems and more complex natural environments may initially seem bleak. However, the tool set for examining these questions is growing with new molecular techniques for tracking infections (Vardo *et al.* 2005; Wargo *et al.* 2006), statistical methods for analysing complex temporal relationships (Zuur *et al.* 2009), and new modelling techniques for linking within and between host processes (Day *et al.* 2011; Mideo *et al.* 2011). Furthermore, recent studies have provided encouragement that the lessons from laboratory experiments with model systems are relevant to natural infections (e.g. Harrington *et al.* 2009) and as interest in the life-history strategies of other parasite species increases there will be opportunities for lessons from other systems to feed back into malaria research.

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Competition and the Evolution of Reproductive Restraint in Malaria Parasites

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ABSTRACT: All organisms must trade off resource allocation between different life processes that determine their survival and reproduction. Malaria parasites replicate asexually in the host but must produce sexual stages to transmit between hosts. Because different specialized stages are required for these functions, the division of resources between these life-history components is a key problem for natural selection to solve. Despite the medical and economic importance of these parasites, their reproductive strategies remain poorly understood and often seem counterintuitive. Here, we tested recent theory predicting that in-host competition shapes how parasites trade off investment in in-host replication relative to between-host transmission. We demonstrate, across several genotypes, that *Plasmodium chabaudi* parasites detect the presence of competing genotypes and facultatively respond by reducing their investment in sexual stages in the manner predicted to maximize their competitive ability. Furthermore, we show that genotypes adjust their allocation to sexual stages in line with the availability of exploitable red blood cell resources. Our findings are predicted by evolutionary theory developed to explain life-history trade-offs in more traditionally studied multicellular taxa and suggest that the answer to the long-standing question of why so few transmission stages are produced is that in most natural infections heavy investment in reproduction may compromise in-host survival.

Keywords: phenotypic plasticity, *Plasmodium*, transmission, survival, life history, trade-off.

Introduction

Explaining variation in the life-history traits exhibited by individuals is a major aim in evolutionary biology. Life-history theory provides a solid foundation for understanding plasticity in resource allocation trade-offs and can be used to explain and predict the evolutionary consequences of environmental variation (Roff 1992; Stearns 1992). There is also increasing interest in using an evolutionary approach to understand how parasite life-history traits shape within-infection dynamics and contribute to virulence and transmission (e.g., Eisen and Schall 2000; Day 2003; Paul and Brey 2003; Paul et al. 2003; Foster 2005; Reece et al. 2009). For organisms such as malaria (*Plasmodium*) parasites, in which in-host replication and between-host transmission are achieved by different specialized stages, the division of resources between these life-history components is a key problem for natural selection to solve. This is analogous to the trade-off between reproduction and maintenance faced by multicellular sexually reproducing organisms (Koella and Antia 1995). The assumption that reproduction is costly, resulting in trade-offs between reproduction and survival and between current and future reproductive effort, is a key concept in evolutionary biology (Williams 1966).

Life-history theory has provided a wealth of predictions for how the best solutions to resource allocation trade-offs are influenced by the state of individuals and the environments they experience (Williams 1966; Roff 1992; Stearns 1992; Pigliucci 2001). However, precisely how individuals should adjust their allocation decisions when resources become scarce is unclear. A decrease in the availability of resources can select for increased investment in maintenance (survival), which is achieved by reducing reproductive effort ("reproductive restraint"; Fischer et al. 2009; McNamara et al. 2009). Conversely, reduced survival probability can favor increased investment in reproduction (in the extreme, "terminal investment"; Williams 1966; Charlesworth and Leon 1976; Fischer et al. 2009). These investment decisions may lie at opposite ends of a continuum, and whether individuals can adopt the best solution depends on the costs and constraints of plasticity and the accuracy of available information. Testing how individuals trade off investment between survival and re-

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production has been constrained by difficulties in measuring reproductive effort for multicellular organisms (Clutton-Brock 1984; but see Creighton et al. 2009). Reproductive effort can be readily measured for malaria parasites; however, despite more than a century of research into interventions that block reproduction, their investment strategies remain poorly understood (Reece et al. 2009). Previous studies suggest that when in-host survival is threatened parasites increase investment in between-host transmission at the expense of in-host replication (Buckling et al. 1997, 1999*b*; Poulin 2003; Stepniewska et al. 2004; Bousema et al. 2008; Peatey et al. 2009), but recent evolutionary theory predicts that the opposite should occur (Mideo and Day 2008; Mideo 2009).

As a general rule, malaria parasites appear to invest remarkably little in transmission throughout infections (Taylor and Read 1997); explanations for this apparent reproductive restraint include reducing virulence experienced by vectors, minimizing the extent to which hosts develop transmission blocking immunity, and using numerically dominant asexual stages to shield transmission stages from attack by nonspecific immune factors (Taylor and Read 1997; McKenzie and Bossert 1998). However, recent formal theory predicts that within-host competition for resources alone can be sufficient to select for reproductive restraint (Mideo and Day 2008). Genetically mixed infections are common (Babiker et al. 1991; Mayxay et al. 2004; Vardo and Schall 2007), so parasites frequently interact with coinfecting genotypes (including con- and heterospecifics). That competition results in suppressed growth and transmission of coinfecting genotypes is well known, but the underlying contributions of exploitation (e.g., for red blood cell resources) and apparent (immune-mediated) competition are unclear (Taylor et al. 1997*a*; Haydon et al. 2003; Mayxay et al. 2004; Bell et al. 2006; Råberg et al. 2006; Barclay et al. 2008). Theory predicts that parasites experiencing local competition (within the host) should divert investment away from reproduction to maximize their ability to replicate and thus exploit the greatest share of red blood cell resources. The recent discovery that malaria parasites can detect and respond to the presence of unrelated competitors (Reece et al. 2008) suggests that they could also use this information to decide how much to invest in reproduction. Previous attempts to test this prediction have been inconclusive (Taylor et al. 1997*b*; Wargo et al. 2007*b*; Bousema et al. 2008), but these studies did not consider patterns of reproductive effort or performance of focal genotypes in mixed infections.

Here, we used several genotypes of the rodent malaria parasite *Plasmodium chabaudi* to test whether parasites facultatively respond to in-host competition by decreasing their investment in between-host transmission. We investigated how competition, resource availability, and genetic variation interact to shape patterns of investment in gametocytes during infections. First, we used a bank of genotypes to test for genetic variation in patterns of gametocyte investment throughout infections. Second, we monitored three focal genotypes in single and mixed infections with one or more competitors to test whether investment in gametocytes is facultatively reduced in competition. Third, we predicted that if reproductive restraint in mixed infections enables parasites to gain the greatest share of exploitable resources, then the investment decisions of each genotype will be influenced by the availability of these resources.

Methods

Infections and Experimental Design

We used *Plasmodium chabaudi* genotypes from the World Health Organization Registry of Standard Malaria Parasites (University of Edinburgh). These wild-type clonal genotypes were isolated from an area where mixed infections are frequent (Carter 1978). Infections described in this article were originally set up to examine sex ratios in single and mixed infections. Full details are available in Reece et al. (2008). Briefly, the treatment groups were as follows: (1) six groups of single-genotype infections, consisting of 1×10^6 AJ, AS, ER, CR, CW, or DK parasites; (2) two groups of two-genotype infections, one group with 1×10^6 AJ + 1×10^6 AS parasites and a second group with 1×10^6 AJ + 1×10^6 ER parasites; and (3) one group of three-genotype infections with 1×10^6 AJ + 1×10^6 AS + 1×10^6 ER parasites. Although the starting doses of parasites were higher in mixed infections than in single infections, the starting dose of each focal genotype was kept constant. This was necessary because comparing the behavior of genotypes in different scenarios requires initiating infections of focal genotypes in the same way and manipulating only the in-host environment they experience (i.e., the presence or absence of competition) for each treatment. Previous experiments have demonstrated that variation in the number of parasites initiating infections has negligible effects on infection dynamics. Specifically, varying starting densities from 1×10^2 to 1×10^8 parasites (for comparison, our infections varied from 1×10^6 to 3×10^6) had very little effect on asexual production during infections (Timms et al. 2001) and, more importantly, on gametocyte production (Timms 2001). Thus, our experimental design is standard in studies examining the effect of in-host competition in malaria (including de Roode et al. 2005*a*; Råberg et al. 2006; Reece et al. 2008; Wargo et al. 2007*b*) and has also been used for other systems (e.g., Balmer et al. 2009; Lohr et al. 2010).

We used polymerase chain reaction assays (Drew and Reece 2007) to distinguish and quantify the asexual stages and gametocytes produced by each clone throughout infection. Our assays enabled us to monitor each competitor in the two-genotype infections, which maximized the power of our analyses while minimizing the number of mice required; however, because AS and ER cannot be distinguished, we monitored only AJ in three-genotype infections.

We used 6–8-week-old male MF1 mice (in-house supplier, University of Edinburgh), and all treatment groups contained five mice. Sampling was performed in the morning so that circulating parasites were in ring or early-trophozoite stages and to obtain samples before DNA replication for the production of daughter progeny occurs. We restricted our analysis to data collected between patency of infection (day 5 postinfection [PI]) and day 12 PI because (1) it minimized the time for the development of strain-specific immunity (Quin and Langhorne 2001; Achtman et al. 2007) that might kill gametocytes and potentially confound estimates of parasite investment decisions; (2) it maximized power for the analysis, given that three mice in the treatment group with the most virulent strain combination (AJ + ER) died on day 12 PI; and (3) the effects of competition are strongest during the acute phase (Bell et al. 2006). Red blood cell densities were estimated using flow cytometry (Coulter Counter, Beckman Coulter; see Ferguson et al. 2003), and reticulocyte densities were estimated from thin blood smears. All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

Gametocyte Conversion Rate

Competitive suppression in mixed infections is well documented (Taylor et al. 1997*a*; Paul et al. 2002; de Roode et al. 2004*b*, 2005*a*, 2005*b*; Bell et al. 2006; Råberg et al. 2006) and results in a reduction in the densities of all competing genotypes (relative to those achieved in single infections). Because suppressed genotypes have a smaller pool of parasites from which to produce gametocytes, simply observing lower densities of gametocytes in mixed infections would not reveal whether parasites had invested a lower proportion of their number into developing as sexual stages. Similarly, variation in gametocyte density could be generated from the same relative level of investment by cohorts that simply differ in parasite number. Therefore, we calculated the conversion rate (following Buckling et al. 1999*b*), which is the standard method for measuring investment in sexual stages (gametocytes). The conversion rate represents the proportion of parasites in a given synchronous cohort that differentiate into sexual stages relative to asexual stages and takes into account the growth rate for each genotype and the time asexual stages and gametocytes take to mature (24 and 48 h, respectively) in our *P. chabaudi* model system.

Analyses

We used R software (version 2.6.1; R Foundation for Statistical Computing; http://www.R-project.org) for all analyses. We subjected conversion rates to arcsine square root transformation and performed analyses using linear mixed-effect models with mouse as a random effect to overcome pseudoreplication problems of repeated sampling of infections in each host. We followed model simplification by sequentially dropping the least significant term and comparing the change in deviance with and without the term to χ^2 distributions until the minimal adequate model was reached. Degrees of freedom corresponded to the difference in the number of terms in the model. For analyses reported in "Resource Availability," the density of available uninfected red blood cells was calculated by deducting the daily total parasite density from the corresponding daily density of red blood cells.

Results

Gametocyte Investment during Infections

We monitored six wild-type genotypes (AS, AJ, ER, DK, CR, and CW) of the rodent malaria parasite *Plasmodium chabaudi* to test whether there is within-species genetic variation for conversion rates during the acute stage of infection. Each genotype followed significantly different patterns of conversion across the course of its infections (genotype × day PI: $\chi^2_{35} = 111.05$, $P < .0001$; fig. 1). Genetic variation in a trait is required for selection to shape its expression and could reflect differences in resource acquisition abilities. Therefore, we tested whether conversion rates are related to the abundance and age of available red blood cell resources. The resources available to each parasite cohort explained significant variation in conversion rates, with positive correlations between conversion rate and red blood cell density for five of the six genotypes ($\chi^2_5 = 11.73$, $P = .039$) and the proportion of red blood cells that were reticulocytes (young red blood cells; $\chi^2_1 = 4.42$, $P = .036$) for all genotypes.

Effect of Competition on Gametocyte Investment

We used three genotypes (AS, AJ, and ER) to test whether conversion rates are adjusted in response to competition. For each genotype, we compared the conversion rates produced in single infections to those produced in competition with different numbers of unrelated conspecifics.

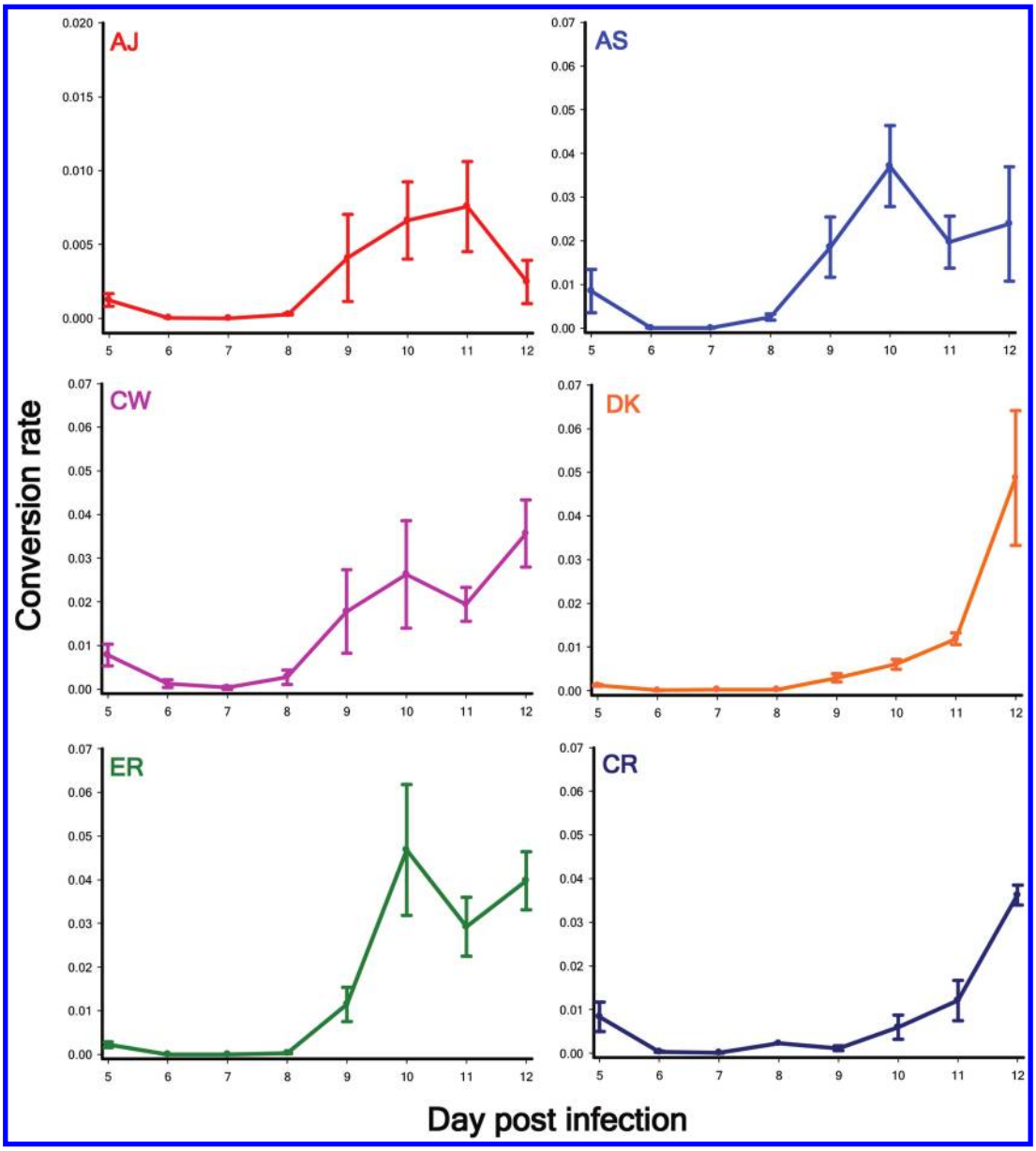

Figure 1: Genetic variation for investment in reproduction (transmission stages). Shown is the production of gametocyte stages relative to asexual stages (conversion rate) for six wild-type *Plasmodium chabaudi* genotypes during the acute stage of infection. Error bars show the SEM for five independent infections per genotype.

Because our quantitative polymerase chain reaction assays could distinguish AJ asexual stages and gametocytes from those of AS and ER but not between AS and ER (Drew and Reece 2007), we monitored AS and ER in double infections (each with AJ) and AJ in double and triple infections (with AS, ER, or both). The conversion rates produced by AJ did not significantly differ depending on the identity (day PI × competitor genotype: $\chi^2_8 = 6.15$, $P > .5$) or number (day PI × competitor number: $\chi^2_9 = 7.78$, $P > .5$) of coinfecting genotypes; therefore, we simplified our analysis to two treatment conditions, "alone" and "in competition," for each of the three focal genotypes.

The existence of competitive suppression is required for our experimental manipulations, and the asexual densities for all focal genotypes were significantly reduced in mixed infections (genotype × day PI × treatment: $\chi^2_{14} = 56.22$, $P < .0001$; fig. 2*C*). As predicted, compared with

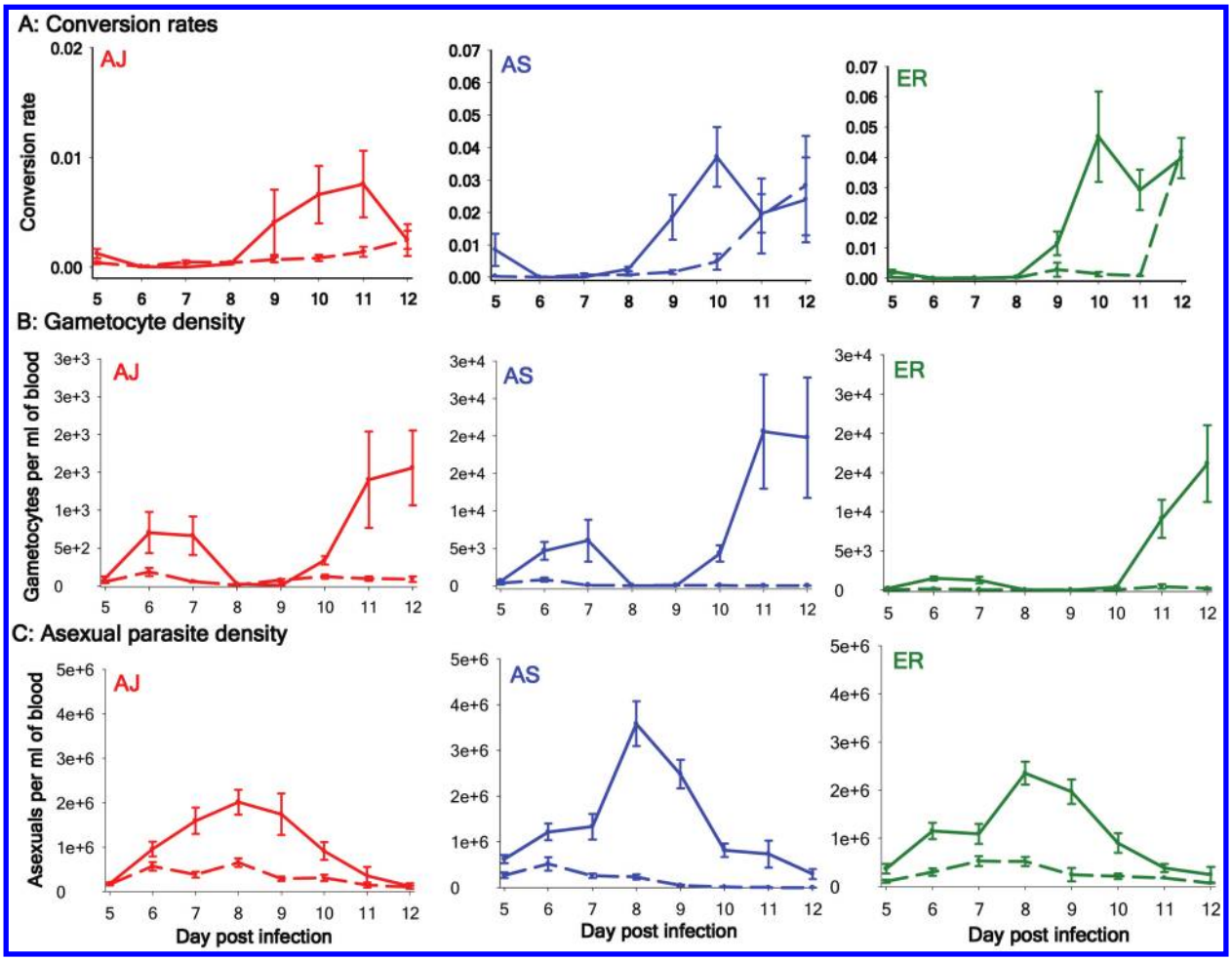

Figure 2: Analysis of competitive suppression. Competitive suppression of parasites led to reduced investment in reproduction (transmission) and lower gametocyte density. Shown are the conversion rate (*A*), gametocyte density (*B*), and asexual parasite density (*C*) for three focal genotypes alone (*solid lines*) and in competition (*dashed lines*). Error bars show the SEM for between five and 15 independent infections per group.

their behavior in single infections (alone), all three genotypes significantly reduced their conversion rates in mixed infections (in competition), and the magnitude of this effect varied over the course of infections and between the genotypes (genotype × day PI × treatment: $\chi^2_{14} = 39.59$, $P < .0005$; fig. 2*A*). Lower gametocytes densities were also observed for each focal genotype in mixed infections (genotype × day PI × treatment: $\chi^2_{14} = 59.08$, $P < .0001$; fig. 2*B*). In addition to the effect of competition, conversion rates also had a significant negative correlation with the overall densities of parasites in infections ($\chi^2_1 = 10.56$, $P < .005$), but there was no significant correlation between conversion rates and red blood cell density ($\chi^2_1 = 0.03$, $P = .87$) or the proportion of red blood cells that were reticulocytes ($\chi^2_1 = 2.58$, $P = .11$).

Resource Availability

Because our single-infection data suggested that gametocyte investment is related to the availability of red blood cell resources but our competition data suggested that competition per se is more important, we investigated whether resources influence the strategies of competing parasites in more depth. We focused on the data from mixed infections to test whether the conversion rate for each parasite cohort correlated with the density of available red blood cells. Because we found no significant difference in the conversion rates for AJ and ER ($\chi^2_3 = 3.66$, $P > .1$), we simplified our analysis to group these genotypes together. For all genotypes, there was a positive correlation between the density of available red blood cells and con-

version rate, and this effect was greater for AS than for AJ/ER (genotype × resource availability: $\chi^2_1 = 12.01$, $P < .0005$, AS estimated slope $= 4.46 \times 10^{-5}$ [$\pm 1.26 \times 10^{-5}$], AJ/ER estimated slope $= 4.08 \times 10^{-6}$ [$\pm 5.42 \times 10^{-7}$]).

Discussion

Testing the assumption that reproduction is costly and trades off against investment in survival is a challenge in evolutionary biology because of potentially confounding effects of differential survival of individuals that vary in quality, multiple components of reproductive investment (e.g., egg provisioning and parental care), decline through senescence, and performance improvement as individuals gain experience. Malaria parasites do not have these complexities and genotypes can be tested in different environments, thus providing a novel system with which to test life-history theory (Reece et al. 2009). Our analyses reveal significant patterns of genetic variation and phenotypic plasticity (figs. 1, 2) in the reproductive effort of malaria parasites. Our experiment also demonstrates, across several genotypes, that investment in gametocytes is facultatively reduced when parasites experience mixed infections (fig. 2) and that this response is influenced by resource availability.

Adopting a reproductive-restraint strategy in competition (fig. 2) is predicted to enable parasites to maximize their share of exploitable resources in mixed infections (Mideo and Day 2008). Despite the widespread occurrence of genetically mixed infections in natural populations, how in-host competition shapes plasticity in parasite life-history trade-offs is rarely considered (but see Wargo et al. 2007*b*). Parasites in mixed infections experience competitive suppression, which could be due to direct (exploitation) competition for resources such as red blood cells (Mideo and Day 2008) or to apparent competition in which the presence of another genotype results in cross-reactive immune responses that are damaging for both genotypes (McKenzie and Bossert 1998). Diverting investment away from gametocytes and into asexual replication, as observed in our experiment, benefits parasites experiencing both types of competition. The "safety in numbers" afforded by reproductive restraint may enable parasites to withstand attack from cross-reacting immune responses as well as maximize the ability to compete for resources. Interactions between these different forms of competition are well known in predator-prey ecology; the presence of a competitor can both reduce food availability and increase predator numbers (Holt 1977; Jones et al. 2009). Disentangling the relative influences of exploitation and apparent competition is challenging. Our experiment was not designed to test for apparent competition, but our mixed-infection data suggest that exploitation competition plays a role, given that conversion rates were positively correlated with the availability of red blood cell resources.

Reducing investment in gametocytes as a response to competition is predicted to maximize competitive ability, but the parasites in our experiment—like all other mixed-infection experiments with this model system—still experienced suppression (fig. 2*C*). Does this suggest that the parasites did not benefit from adopting reproductive restraint, or would they have been even more suppressed without this strategy? Quantifying the effects of reproductive restraint on competitive ability requires comparing the extent of suppression experienced by parasites that can and cannot reduce their gametocyte investment. Measuring the fitness consequences of investment decisions is difficult, but one way to achieve this would be through identifying the mechanism used to detect coinfecting competitors and manipulating parasites to be unable to respond. The costs and benefits of altering gametocyte investment will also depend on aspects of parasite ecology and population biology. For example, parasites restricted to infecting a specific age class of red blood cells may face more severe resource limitation in competition than do more generalist parasites (Reece et al. 2005). Resource limitation may occur during periods of anemia, but for parasites that can infect reticulocytes anemia may signal an imminent influx of resources, making reproductive restraint the best short-term solution. Another important aspect of the trade-off between current and future reproduction concerns opportunities for transmission to mosquitoes and the type of hosts to which parasites will be transmitted (Day 2002; Alizon and van Baalen 2008; Reece et al. 2009). For example, in-host survival may be sufficiently important to constrain gametocyte investment to low levels in areas with irregular or low transmission or if there is a high probability that the next host already harbors competing parasites (Vardo et al. 2007).

In contrast to our data, most previous studies have demonstrated that parasites do not adopt reproductive restraint when experiencing stress. For example, parasites are known to produce more gametocytes when exposed to subcurative doses of antimalarial drugs, anemia, or changes in the age of available red blood cells (Trager and Gill 1992; Buckling et al. 1997, 1999*a*; Reece et al. 2005). Most explanations assume that these changes in the in-host environment cause sufficient reduction in parasite survival to induce terminal investment in reproduction (Buckling et al. 1997, 1999*a*; Stepniewska et al. 2004; Peatey et al. 2009). However, reproductive restraint has now been observed in response to low doses of drugs (Reece et al. 2010), and the influx of reticulocytes during anemia may benefit parasites that can use this resource (Reece et al. 2005). Therefore, we suggest that changes in the in-host environment should be evaluated in the context of

parasite ecology to interpret whether changes in gametocyte investment are due to reproductive restraint, terminal investment, or a response to increased resources. More broadly, the ability of parasites to fine tune gametocyte investment in response to subtle changes in their in-host environment highlights the importance of measuring and accounting for variation in these parameters when investigating the effects of experimental manipulations on parasite behavior.

Few studies (but see Buckling et al. 1999*b*; Wargo et al. 2007*b*) have formally tested whether genetic variation in patterns of gametocyte densities are due to different investment strategies or are simply by-products of variation in asexual stage densities. By using a bank of six genotypes and monitoring infections initiated with the same starting dose in immunologically naive hosts, we have demonstrated genetic variation in reproductive investment (fig. 1). The explanations for this variation are not yet known; investment patterns are not related to the virulence rankings of the genotypes (Mackinnon and Read 1999) or to competitive ability (Bell et al. 2006). Alternatively, there may be genetic variation for the range of red blood cell ages that parasites can invade and utilize (Antia et al. 2008; Mideo et al. 2008). However, while our data revealed that gametocyte investment is positively correlated with the abundance of mature and immature red blood cells, we did not find any G × E interactions for this pattern. Because *Plasmodium chabaudi* is in general able to infect all ages of circulating red blood cells, all genotypes may simply be able to afford greater investment in gametocytes when resources are abundant (Reece et al. 2005).

To investigate whether resource availability matters in competition, we focused only on the mixed infections and asked whether the conversion rates for each cohort correlated with the density of uninfected red blood cells. We predicted that gametocyte investment would be positively correlated with available resources. Data on all three of our genotypes (AS, AJ, and ER) supported this prediction and demonstrated genetic variation in these patterns because the slope for this correlation was greater for AS than for AJ and ER. Why this genetic variation exists is unclear, but it could be related to competitive ability given that AJ and ER are known to be superior competitors relative to AS (Bell et al. 2006). This could be tested by initiating infections with competing genotypes at different densities and frequencies in hosts with manipulated levels of anemia to span a broader range of competitive suppression and resource availability. Such experiments, ideally combined with modeling, could also reveal whether decisions span the continuum from reproductive restraint to terminal investment and how parasites respond to different stresses that affect the proliferation rate.

Explaining how parasites respond to changes in their in-host environment is important for understanding patterns of virulence and transmission. All else being equal, when parasites decrease investment in gametocytes, relatively more of the host-damaging asexual stages are produced. Thus, by most measures a strain that shifts investment away from gametocytes is likely to be more "virulent" (Mideo and Day 2008). In light of this, our results are also in agreement with a large body of theory on virulence evolution predicting that in mixed infections parasites are selected to become less prudent (Frank 1994, 1996). Furthermore, if medically relevant parasite species adopt reproductive restraint in response to in-host competition, selection could be strong enough to fix parasite investment in gametocytes at a low level where mixed infections are common. If so, these parasites would have a greater capacity to cause disease when released from competition—for example, as a result of evolving drug resistance and the removal of sensitive competitors by drug treatment (de Roode et al. 2004*a*; Wargo et al. 2007*a*).

Translating the results from our model system to human malaria parasites, especially *Plasmodium falciparum*, may be complicated by differences in the traits that underlie virulence. For example, in contrast to *P. chabaudi*, rosetting is a virulence trait in *P. falciparum*, but occurrence of this phenotype also correlates with parasitemia (Rowe et al. 2002). If *P. falciparum* responds to competition in the same way as *P. chabaudi*, we expect hosts to harbor infections made up of fewer gametocytes relative to asexual parasites in regions of endemicity, where mixed infections are frequent. There is some evidence that *P. falciparum* gametocyte densities are lower in areas of endemicity and in older individuals (Drakeley et al. 2006). A proposed explanation for these trends is that immunity develops more rapidly to gametocytes than to asexual stages and that stronger responses develop in areas of endemicity and in older patients (Drakeley et al. 2006). However, we suggest a possible alternative explanation—that infections in areas of endemicity and older hosts are more likely to contain a mixture of genotypes. Now that tools to measure genetic diversity in natural infections are available, it should be possible to test these hypotheses.

Reciprocal competitive suppression is well known in our rodent malaria system (Taylor et al. 1997*a*; Paul et al. 2002; de Roode et al. 2004*b*, 2005*a*, 2005*b*; Bell et al. 2006; Råberg et al. 2006), but interactions between coinfecting clones may be more complex in *P. falciparum* infection. Parasite interactions may span from facilitation (e.g., via changes in the age structure of red blood cells; McQueen 2006), to no effect on each other, to direct competition for resources (e.g., red blood cells; de Roode et al. 2005*a*), and these interactions can be intensified or alleviated by cross-reactive immune responses (Gupta et al. 1994; Gil-

bert et al. 1998; Barclay et al. 2008). For example, molecular data suggest that proportions of different clones within mixed infections can fluctuate over time, suggesting that multiple clones are not always present in the circulation (Babiker et al. 1998). This may also mean that whether a clone appears to be a good or a poor competitor is context dependent. Understanding these interactions in natural settings is a huge challenge, but given that there is much variation in the population ecology of human malaria parasites, they offer an attractive system for the study of mixed infections.

In conclusion, the gametocyte investment patterns we observed are predicted by evolutionary theory for life histories and demonstrate that parasites alter their investment in in-host replication (survival) relative to that in between-host transmission (reproduction) in response to competition. That competitive ability and resource abundance also mediate this trade-off adds considerable support to the hypothesis that parasites evaluate their social and in-host environments and respond adaptively (Reece et al. 2009). Our findings also suggest that the answer to the long-standing question of why so few transmission stages are produced (Taylor and Read 1997) is that in most natural infections the importance of investing in within-host survival constrains parasites to low investment in reproduction. The extent to which individuals display phenotypic plasticity in their reproductive effort depends on the fitness benefits of different life-history strategies, the costs and limits of assessing the relevant environmental or internal parameters, and constraints on the range of investment strategies that can be adopted (de Witt et al. 1998). If the proximate mechanisms underlying phenotypic plasticity in malaria parasites can be identified, it may be possible to manipulate their behavior in clinically and epidemiologically beneficial ways.

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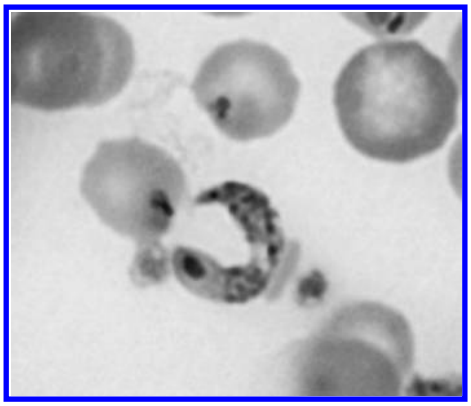
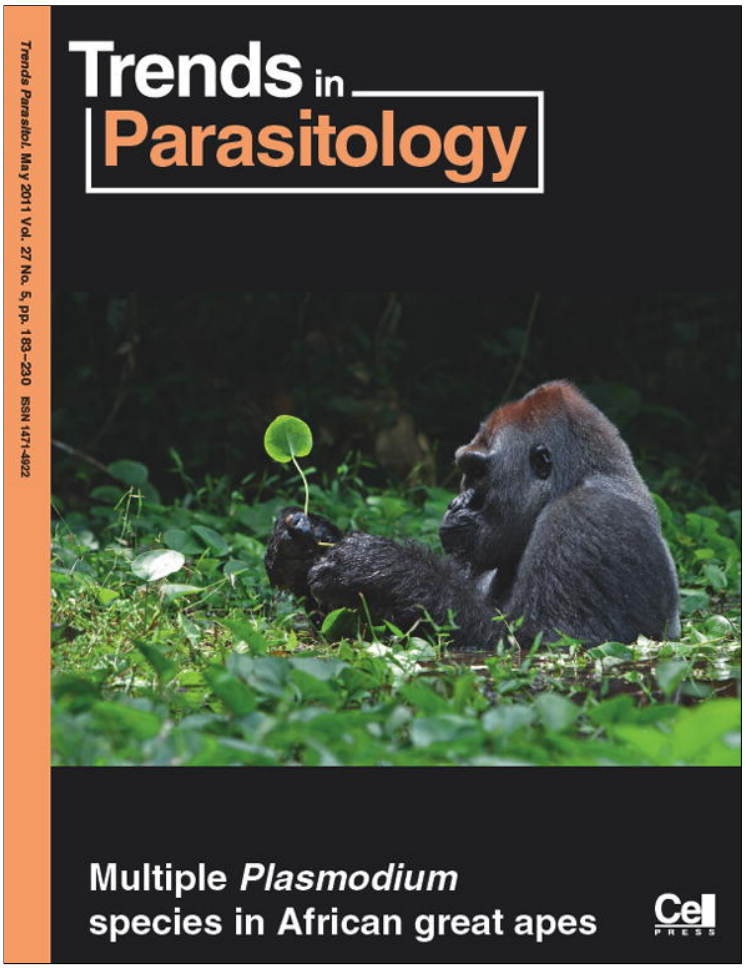


Malaria parasite transmission form in the blood. Photograph by Sarah Reece.



Opinion

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Malaria and trypanosome transmission: different parasites, same rules?

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African trypanosomes produce different specialized stages for within-host replication and between-host transmission and therefore face a resource allocation trade-off between maintaining the current infection (survival) and investment into transmission (reproduction). Evolutionary theory predicts the resolution of this trade-off will significantly affect virulence and infectiousness. The application of life history theory to malaria parasites has provided novel insight into their strategies for survival and reproduction; how this framework can now be applied to trypanosomes is discussed. Specifically, predictions for how parasites trade-off investment in survival and transmission in response to variation in the within-host environment are outlined. An evolutionary approach has the power to explain why patterns of investment vary between strains and during infections, giving important insights into parasite biology.

Protozoan parasites: life history trade-offs

Protozoan parasites, such as African trypanosomes (*Trypanosoma brucei* sp.) and malaria parasites (*Plasmodium* sp.), cause serious mortality and morbidity in humans, livestock and wildlife and have severe economic impacts in the developing world. These parasites undergo asexual replication within a vertebrate host and must produce specialized transmission stages to be transmitted between hosts by insect vectors. Evolutionary theory predicts that this life cycle results in a trade-off between the investment of resources into survival (replication) and reproduction (production of transmission stages; Box 1). Survival versus reproduction trade-offs are a key concept in evolutionary biology and have received a wealth of theoretical and empirical attention [1]. Whereas most of the concepts of life history theory have been developed for multicellular organisms, parasites face similar challenges; species competing for resources within a host and being targeted by the immune response are analogous to prey species competing for food and avoiding predators [2,3]. The predictions of theory are being met with increasing support across a diverse range of taxa [4,5], including single-celled parasites [4,6–8].

In recent malaria research, life history theory has provided insight into how parasites respond to selection pressures, such as co-infection with other genotypes or species, or attack from anti-malarial drugs [4,9–11]. This framework has been successful in explaining the patterns observed in laboratory experiments with model systems [11–14], and there is also some evidence that these findings are relevant to human malaria parasites in natural infections [15] and *in vitro* studies [16]. By contrast, trypanosome research has largely remained focused on molecular and cellular biology (but see [17]). The success of using life history theory to understand the strategies of malaria parasites suggests that this framework can also be applied usefully to trypanosomes to explain variation in parasite strategies, across genotypes and during infections. This article discusses how predictions from life history theory can be applied to understand the investment strategies of trypanosomes. The trade-off between investment in survival (replication) and reproduction (production of transmissible stumpy forms) is focused on for two reasons. First, there are clear and useful parallels with recent findings in malaria parasites (Box 2). Second, the relative investment in within-host replication and between-host transmission is predicted to have significant effects on virulence and infectiousness [18].

Trypanosomes: survival and reproduction

When an infected tsetse fly bites a mammalian host, metacyclic forms are inoculated into the blood. These develop into slender form parasites that undergo rapid asexual replication, maintaining the infection in the host (survival). As parasite density increases, a parasite-derived factor accumulates (termed stumpy induction factor, SIF) and causes some, but importantly not all, parasites to undergo cell cycle arrest and differentiate into stumpy forms [19]. Stumpy forms have a limited life expectancy in the blood because they no longer replicate or productively switch their VSG coat, but they are infective to tsetse flies and therefore provide the potential for transmission (reproduction) [20]. Trypanosome infections generally involve cyclical peaks in parasitaemia (Figure 1). Statistical modelling indicates that parasite driven differentiation, together with antigenic variation, can generate this pattern, and therefore the distinctive waves of parasitaemia are predominately under parasite control [21]. The role that differentiation plays in generating waves of parasitaemia is supported by observations that laboratory strains that cannot produce stumpy

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Box 1. Key concepts in evolutionary ecology

Life history trade-offs

Life history traits are phenotypic components influencing fitness [46]. For parasites this includes replication rate and the relative investment into and timing of producing transmission stages [4]. Whereas organisms are selected to maximize fitness, they are also constrained by trade-offs between different life history traits [46]. Trade-offs can take various forms but the most commonly considered result from resource limitation: organisms have limited resources to invest in different life processes and therefore need to balance investment to maximize fitness [46]. In metazoans, single organisms are easily identifiable as the target of natural selection. In single-celled parasites, a genotype within an infection is the comparable target [7,26]. When infections consist of a single genotype, trade-offs will be resolved across all parasites in the host, maximizing overall fitness over the lifetime of the infection [7]. An important trade-off, especially in long lasting infections such as with malaria and trypanosome parasites, is between current investment in between-host transmission and investment in maintaining the infection (within-host survival) for future transmission [4,28].

Phenotypic plasticity and fixed strategies

Examining and explaining trade-offs is complicated due to organisms evolving under varying environmental conditions. The best solutions to resource allocation trade-offs depend on the opportunities and constraints offered by the within-host environment and how they change throughout infections [4,32].

Environmental conditions can lead to changes in life-history traits by two distinct, but not mutually exclusive, processes [32,47]. First, organisms might be able to produce a range of phenotypic responses according to variation in environmental or internal conditions. This process, known as adaptive phenotypic plasticity, is central to understanding the effects of environmental variation on evolution and can be broadly defined as a change in the phenotype of a given genotype in response to environmental cues [48]. This enables organisms to respond rapidly to predictable environmental changes in ways that maximize fitness [32]. For example, the freshwater crustacean *Daphnia pulex* produces costly morphological defences, including neck spines, when exposed to predator cues [49]. Second, with longer-term environmental changes, spanning multiple generations, microevolution can occur where population gene frequencies change because of individuals best adapted to the new conditions disproportionably contributing to future generations [32]. For example, when a new high coverage drug treatment is introduced, genes for resistance mechanisms spread in the population of parasites targeted [50].

Plasticity and microevolutionary processes can work together to shape the genotypes and the phenotypes organisms display [32,48]. Organisms that are likely to encounter a range of environmental conditions use plasticity to match their phenotype to changes in their circumstances. But maintaining mechanisms to detect, process, and respond to environmental cues is costly and organisms also risk inaccurate cues leading to the wrong phenotype [4,51]. For these reasons different degrees of plasticity will occur in natural infections, and if environmental changes stabilize, phenotypic plasticity may be replaced by fixed strategies [4].

forms continue to replicate, quickly killing the host [22], and that cycles of parasitaemia are still observed in infections of immunocompromised mice [23].

However, in natural infections, parasitaemia will be shaped by a combination of the host immune response and the production of stumpy forms. To evade the host immune response, trypanosomes employ a strategy of changing their variant surface glycoprotein (VSG) surface coat. Each parasite has a repertoire of thousands of VSG genes but expresses only one at a time [24]. Initially in laboratory infections, one or a few VSG variants dominate but the immune system eventually raises antibodies against these coats, leading to wide-scale clearance. Each parasite has a low probability of switching to the expression of a new variant [25]. Therefore, during every replication cycle, a small number of parasites probably have a VSG coat not yet recognized by the immune system, and these parasites will rapidly replicate, resulting in a new wave of parasitaemia (Figure 1) [25]. It is important to note that although variants differ in the VSG gene(s) being expressed over the course of a single infection, they are isogenic to the original infecting parasite clone(s). This is significant because natural selection acts at the level of the parasite genotype within infections, therefore clonally related parasites will be selected as a group to maximize the transmission of their genotype over the course of the infection [7,26].

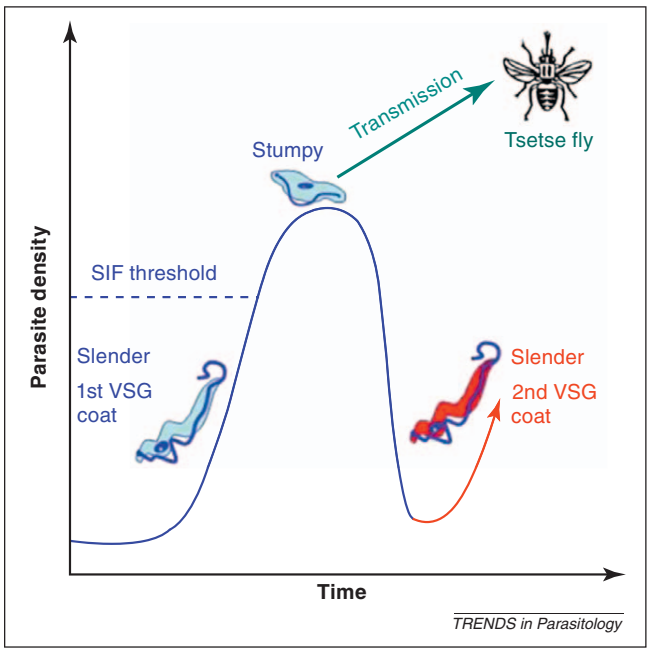

Figure 1. Dynamics of trypanosome infection in the mammalian host. As slender form parasites replicate in the blood, the parasitaemia rises, as does the concentration of a soluble stumpy induction factor (SIF), inducing some parasites to differentiate into non-replicating, but transmissible, stumpy forms. A combination of differentiation into stumpy forms and clearance, as the immune system mounts a response to the first VSG coat, leads to a crash in parasitaemia. However, because some slender forms have switched VSG coats, a second wave of parasites, not yet recognized by the immune system, begins to increase parasitaemia once again.

Each trypanosome faces a trade-off between differentiation into a transmissible stumpy form and continued division as a slender form. From the perspective of a parasite cohort, continued replication of slender forms is necessary to withstand attack from the immune system; for example, maintaining parasite numbers provides the potential to express new VSG coats, whereas stumpy forms provide the potential for between-host transmission. This trade-off has obvious parallels with gametocyte production in malaria parasites (Box 2). Also, similar to malaria parasites, trypanosomes will experience variation in their within-host environment, both during infections and in different hosts, which is predicted to influence the balance between investment in slender and stumpy forms.

Box 2. Malaria parasite investment strategies in response to stress

Malaria parasites replicate asexually within the red blood cells of their vertebrate host but also produce specialized transmission stages (gametocytes). When male and female gametocytes are taken up in a mosquito blood meal they produce gametes and undergo a round of sexual reproduction to infect the vector. The trade-off faced by malaria parasites between asexual replication and production of gametocytes is analogous to the growth versus reproduction trade-off faced by all sexually reproducing organisms [4]. Getting the right balance is essential; too few transmission stages results in an evolutionary dead end within the host, but too many can lead to the infection being cleared before a transmission opportunity arises.

Mounting evidence from experimental *Plasmodium chabaudi* infections in mice and *in vitro* cultures of *Plasmodium falciparum* show that malaria parasites vary their investment in gametocytes during infections depending on aspects of their environment (Table I). These patterns initially seem contradictory and confusing but can be explained in a life history framework in which parasites are expected to respond to changes in the constraints and opportunities experienced during infections [4]. Malaria parasites increase investment in gametocytes when experiencing either very good or very poor conditions. When conditions are good (e.g. high density of preferred red blood cells), parasites have plenty of resources and can afford to invest in gametocytes [12,52]. When conditions are very poor (e.g. high drug doses or severe resource limitation), then continued survival in the host is unlikely, and parasites make a terminal investment in gametocytes to maximize short-term transmission [53,54]. More commonly, parasites experience intermediate stress levels (e.g. competition with other strains, low levels of drugs or host immune factors), and are constrained to ensuring within-host survival by reducing investment in gametocytes (reproductive restraint) [12,16,28]. It is important to note, however, these patterns will be made more complex by the details of interactions between host and parasite genotypes [29].

A life history framework not only explains variation observed in malaria transmission strategies [12,14,16], but these studies have also provided novel insights into other aspects of their biology. The ability to respond to various aspects of their within-host environment reveals parasite mechanisms to detect information about their surroundings. Most strikingly, malaria parasites respond to both the genetic diversity of their infection and the densities of their own genotype and co-infecting con-specifics [12,14]. This demonstrates an ability to discriminate between kin and non-kin previously thought limited to complex multicellular organisms.

Table I. Malaria parasite transmission strategies and the within-host environment

Malaria species	Data source	Environmental change	Predicted level of stress, quality of within-host environment	Effect on relative investment in transmission	Ref.
P. chabaudi	Experimental infections in mice	Increased resources	Low stress, high quality within-host environment	All six strains studied increased investment in transmission with higher proportions of young red blood cells (reticulocytes) and five of the six and with total red blood cell density.	[12]
P. falciparum	Cultures with drug sensitive strains from natural infections with frequent drug treatment	Exposure to low doses of anti-malarial drugs	Intermediate	Decreased investment in transmission for all three susceptible strains studied.	[16]
P. chabaudi	Experimental infections in mice	Presence of conspecific competitor	Intermediate	Decreased investment under competition for all three of the strains studied.	[12]
P. chabaudi	Experimental infections in mice	Presence of conspecific competitor	Intermediate	Only significant effect was for decreased investment, but this was only observed in one of two host strains for one of two parasite strains	[29]
P. chabaudi	Experimental infections in mice	Exposure to erythropoietin, which signals host anaemia	High stress, low quality within-host environment	Increased investment seen in one strain of *P. chabaudi* but not in one strain of *Plasmodium vinckei.*	[52]
P. chabaudi	Experimental infections in mice	Exposure to high doses of anti-malarial drugs	High stress, low quality within-host environment	Increased investment in both of the two strains studied.	[53]
P. falciparum	Cultures of laboratory strains	Exposure to high doses of anti-malarial drugs	High stress, low quality within-host environment	Increased investment seen across all four strains studied.	[54]

Strategies to maximize survival and reproduction: evolutionary predictions

Trypanosome parasites reach a threshold before some parasites differentiate into transmissible stumpy forms. Evolutionary theory predicts that, in general, the relative level of investment into reproduction will depend on the quality of the environment and that this relation will be U-shaped [1]. For trypanosomes, investment into transmission (differentiation into stumpy forms) should depend on the quality of the within-host environment. Investment in stumpy forms is predicted to be highest under extremely good conditions, when parasites can afford to

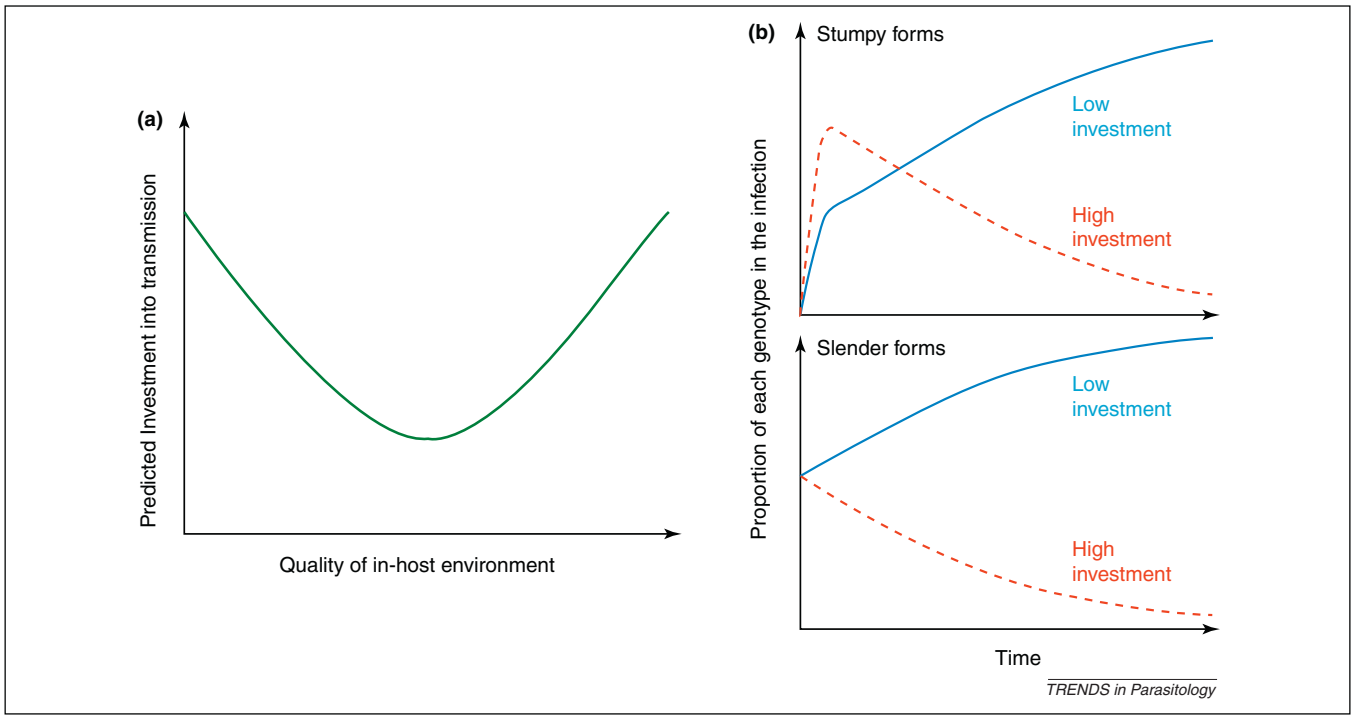

Figure 2. Strategies for the relative investment into transmission stages. **(a)** Theory predicts that organisms will invest heavily in reproduction under either very good or exceptionally poor conditions, and be constrained to investing in survival in intermediate situations [1]. When applied to trypanosomes, parasites are predicted to produce high numbers of transmissible stumpy forms in extremely good or extremely poor within-host environments, but, in most conditions be constrained to producing enough slender form parasites to maintain the current infection. As with malaria parasites, it is probable that there will be genetic variation between strains for the ability to accurately detect and respond to environmental cues, and the level of stress experienced in a given environment [12]. **(b)** When parasites are in mixed infections, differing levels of investment into stumpy forms will influence competitive outcomes. Higher investment in transmission stages (high investment; red dashed line) gives short-term benefits (higher initial rate of transmission) but is detrimental to longer-term success because it is more vulnerable to being cleared. The optimal strategy depends on the duration of infection (chance of being cleared by the immune response or outcompeted and risk of host death) and transmission opportunities for the parasite. For example, in a prolonged mixed genotype infection of trypanosomes, the strain with low investment (blue solid line) has higher fitness because it can transmit for longer.

invest heavily, or extremely poor conditions, where survival is unlikely and parasites employ a strategy of terminal investment. Between these two extremes, parasites will be constrained by investing in within-host survival by adopting reproductive restraint (Figure 2a). The strategies observed at the extremes make intuitive sense, but explaining why reproductive restraint is predicted is more complex. When parasites experience stressful (but not terminal) situations, they must produce enough slender replicating forms to maintain the infection, which lowers the density of transmissible stumpy forms in the short term but maximizes fitness over the course of the infection (Figure 2b). The importance of within-host survival is often overlooked but safe guarding future transmission will be an important determinant of parasite fitness when infections persist over long periods.

Adding ecology

For trypanosomes, like other parasites, key variables determining the quality of the within-host environment include: exposure to immune responses, availability of host resources, exposure to trypanocidal drugs, and the presence of competitors. Trypanosomes live freely within the circulation and generate energy through glycolysis of blood glucose. Although the occurrence of hypoglycaemia, at least at peak parasitaemia, is indicative of it being a limiting resource, the effect of glucose level on trypanosome development *in vivo* is yet to be quantified. Similarly, if the efficacy of drugs (where applied) or the force of attack by the immune system varies, trypanosomes will be exposed to different levels of stress. Competitive suppression has been demonstrated to occur in trypanosomes [17], and clear parallels can be drawn with the responses of malaria parasites to competitors, which are discussed below. In reality, the overall quality of the within-host environment and the net level of stress parasites experience, is likely to be influenced by interactions between these variables and further complicated by both host and parasite factors. However, as a starting point to develop clear predictions that can be tested with laboratory experiments, it is useful to consider these different stresses in terms of where they will place parasites on the axis of environmental quality (Figure 2a).

Within-host competition

Like most organisms, parasites (in genetically mixed infections) encounter competitors, and understanding how this affects parasite traits is receiving attention [4,6,10]. Trypanosomes in mixed infections are suppressed, resulting in lower parasite densities [17]. This could be driven by either resource limitation, mixed infections triggering stronger host defenses, or direct interference competition between strains [10,17,27]. Increasing investment in replication could ameliorate competitive suppression by enabling parasites to exploit the greater share of host resources and/or the generation of new VSG variants. Evolutionary theory for malaria parasites predicts that reproductive restraint maximizes competitive ability [28] and a recent

laboratory study reveals that they employ this strategy (but see [29]) when in competition [12].

The extent of reproductive restraint parasites should adopt is predicted to depend on the extent of suppression, which is determined by relative competitive ability. For a poor competitor, a mixed infection is likely to be a very bad environment because proliferation is heavily suppressed, and a terminal investment could be the best strategy. By contrast, reproductive restraint might be unnecessary for the best competitors who experience the least suppression. These predictions are consistent with observations that malaria parasites with faster replication rates compete more effectively in experimental mixed infections [30] and can be complicated if competitive ability depends on who the competitors are. Furthermore, postponing transmission in the short term to improve competitive ability could be risky if mixed infections are particularly virulent and will probably kill the host rapidly. However, because natural infections of malaria parasites and trypanosomes are usually chronic and persist over multiple replication cycles and competition suppresses overall parasite density, safeguarding future transmission is likely to be an important component of parasite fitness.

Complex within-host environments

Importantly, the quality of the within-host environment will be shaped by multiple interacting factors and will vary over the course of infection. For example, parasite interactions between strains are complex, spanning from facilitation to competitive suppression. These interactions will also influence and be influenced by factors including host immunity and resource availability [10,27,31]. Additionally, intrinsic host factors will also be important, for example, the rate of SIF turnover or immune competence might vary between individual hosts, leading to complex feedbacks with parasite strategies. The relative importance of different factors, such as competition, immunity and resource limitation, in shaping the quality of the within-host environment, and thus precisely where they place parasites on Figure 2a, is yet to be determined. A combination of using mathematical models to explain experimental data and developing evolutionary theory specifically for trypanosomes will be extremely useful.

Responding to environmental change

Parasite investment strategies can be fixed, plastic or a combination of both. Whether parasites evolve fixed or plastic responses to cope with changes in the circumstances experienced during infections depends on: the frequency of encountering situations, the benefits of responding, and the costs and the constraints involved (Box 1) [4,32]. For example, for parasites to plastically alter strategies in mixed infections they must be able to gather information on the genetic diversity of the infection. Bacteria coordinate group behaviours using quorum sensing to transmit and receive information about density and relatedness [33]. Malaria parasites also appear to be capable of responding to density and relatedness, although the mechanism is not yet known [12,14]. Trypanosomes detect and respond to SIF in a density-dependent manner, and there is also evidence for the coordination of group motility behaviours in the tsetse infective (procyclic) form [34].

Given these observations and the extent that the within-host environment varies during infections and between hosts, plastic responses are probable. Trypanosomes could plastically alter investment into stumpy forms by adjusting the amount of SIF produced or their threshold for responding to SIF. Although SIF is yet to be identified [35], experimental work has indicated that it is a small soluble molecule secreted by the replicating slender stages [19]. Adjusting the concentration of circulating SIF could be complicated by variation in rates of host clearance, and whether SIF initiates a response that is strain-specific or pan-infection. Conditioned media produced by one strain was found to be able to induce stumpy form production in two other strains [19], suggesting that SIF could be general across genotypes. Therefore, varying the threshold for responding to SIF might be a better strategy because it could protect parasites from manipulation by co-infecting strains. Laboratory adapted strains become insensitive to the SIF they produce [19]; however, it is not yet known if there is a range of sensitivities or whether it is an 'all or nothing' response.

In parasite populations where mixed infections and the resulting competitive suppression are the norm, reduced investment in transmission is likely to become fixed. This could have dramatic effects on virulence to the host: less virulent strains could actually reduce harm by suppressing more virulent strains [17]. But, as demonstrated for malaria parasites, if these virulent parasites are released from competition (for example, by being the only genotype transmitted, or through selective drug treatment), the brakes would be removed from the replication of the virulent strain and hosts would experience more severe disease [11,15,36].

Where do we go from here?

Life-history theory can provide testable predictions for trypanosome investment strategies. However, to move forward it is necessary to perform controlled and rigorous experiments that examine parasite strategies under manipulated (perturbed) within-host conditions. Because there are clear predictions for how parasites will respond to competition, and mixed infections are a relatively simple experimental manipulation to perform, within-host competition is a good starting point. The integration of mathematical modelling approaches, with experimental data from these experiments, will be crucial to improve our understanding of the complex interactions within infections and their effect on parasite investment strategies. Mathematical models can tease apart the factors and processes underlying biological patterns to form hypotheses that can be tested empirically [37].

Determining the ecology of mixed infections

There has been little work to quantify the prevalence of mixed infections in trypanosome populations, or their influence on parasite phenotypes. However, field research indicates that there is a range of population structures in African trypanosomes [38,39], as well as genetic variation for traits underlying virulence [40]. The genetic tools

available for *Trypanosoma brucei* [41] and large-scale field projects examining the incidence and epidemiology of trypanosome infections could provide a much clearer picture of mixed infections. This requires developing markers to identify, and ideally quantify, different strains. Whereas the ultimate aim will be to understand how the presence of competing genotypes influences trypanosome life history traits and dynamics in natural infections, the first step, as with malaria parasites, will be to perform controlled lab experiments. To do this it will be necessary to increase the number of genetically characterized strains available for experiments. Field strains are available for trypanosomes but are underexploited in experimental settings in favour of laboratory-adapted strains, which although useful for molecular studies, might not provide realistic information on transmission strategies [42].

Quantifying investment into transmission stages

The development of genotype and stage specific qRT-PCR for malaria parasites has made it possible to track focal genotypes during experimental infections to quantify their investment decisions [43,44]. For trypanosomes, classification of cells as slender or stumpy has traditionally depended on their morphological characteristics, an unreliable process because of the existence of intermediate forms. However, a gene array named PAD (proteins associated with differentiation) involved in transmission has recently been identified [45]. Because PAD marks the transmissible stumpy form, assays to quantify its expression will allow researchers to reliably monitor levels of differentiation over the course of the infection [35]. By comparing patterns of investment in transmission stages of focal parasite genotypes, in single and mixed infections, it will be possible to test for plastic responses to competition. Yet, because transmission investment is predicted to be simultaneously influenced by multiple factors (e.g. competition, resource availability, immune responses) as well as variation in their effects on different parasite genotypes, it is important to measure or control for the effects of potentially confounding variables when examining patterns [4]. To understand dynamics in mixed infections it will also be necessary to examine variation in the response to SIF produced by clone mates and other strains across a range of genotypes from areas where mixed infections are common. Again, controlled experiments will be the first step before analysis of samples from natural infections.

Conclusions

Evolutionary ecology can explain parasite traits and uncover strategic (adaptive) patterns in what often seems to be noisy data [4,7]. Trypanosomes provide exciting opportunities for integrating evolutionary biology with parasitology. Because much of their molecular biology is well understood, and there are highly tractable tools for reverse genetic analysis, the mechanisms underpinning parasite traits, such as kin discrimination, can be relatively straightforward to identify. In this way, research into trypanosome life-history strategies can feed back into malaria research where these mechanisms are not yet understood. By explaining parasite life-history traits it will be possible to gain insight into how, when, and why traits underlying transmission and virulence vary, which will lead to better informed control strategies [6].

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Parasites & Vectors

REVIEW Open Access

Investigating the evolution of apoptosis in malaria parasites: the importance of ecology

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Abstract

Apoptosis is a precisely regulated process of cell death which occurs widely in multicellular organisms and is essential for normal development and immune defences. In recent years, interest has grown in the occurrence of apoptosis in unicellular organisms. In particular, as apoptosis has been reported in a wide range of species, including protozoan malaria parasites and trypanosomes, it may provide a novel target for intervention. However, it is important to understand when and why parasites employ an apoptosis strategy before the likely long- and short-term success of such an intervention can be evaluated. The occurrence of apoptosis in unicellular parasites provides a challenge for evolutionary theory to explain as organisms are expected to have evolved to maximise their own proliferation, not death. One possible explanation is that protozoan parasites undergo apoptosis in order to gain a group benefit from controlling their density as this prevents premature vector mortality. However, experimental manipulations to examine the ultimate causes behind apoptosis in parasites are lacking. In this review, we focus on malaria parasites to outline how an evolutionary framework can help make predictions about the ecological circumstances under which apoptosis could evolve. We then highlight the ecological considerations that should be taken into account when designing evolutionary experiments involving markers of cell death, and we call for collaboration between researchers in different fields to identify and develop appropriate markers in reference to parasite ecology and to resolve debates on terminology.

Introduction

Apoptosis is a controlled process of programmed cell death by which unwanted or damaged cells are eliminated [1,2]. In metazoans, the apoptosis pathway was first described over 35 years ago [3], and is now recognised as essential for normal growth and development, as well helping to guard against infections and the onset of cancer [1,4]. The process of apoptosis is initiated by the activation of death receptors, or by intracellular stress conditions [5]. This leads to a series of genetically controlled and ordered biochemical changes, resulting in morphological changes to the cell [5]. These include the condensing of chromatin, DNA breakdown, membrane changes, shrinkage of the cell and finally the formation of apoptotic bodies [6]. The membrane changes involved in apoptosis act as a signal for apoptotic bodies to be taken up by macrophages, preventing inflammation, as well as passing on information to scavenger cells on the cause of death [7]. In mammals, the process of apoptosis is rapid, removing cells within hours of initiation without evoking the inflammatory arm of the immune system.

Traditionally, apoptosis was thought of as a cellular activity exclusively relevant to multicellular organisms, but this view has recently been challenged. Morphological changes during cell death that are consistent with programmed cell death (PCD) have been reported for a range of unicellular organisms, including protozoan parasites [8-12]. The number of studies revealing PCD markers in unicellular organisms is rapidly increasing, and range across bacteria [13], slime moulds [14], yeast [15,16], algae [17], Trypanosomes [18-21], *Leishmania* [18,22], and *Plasmodium* [23-26]. The occurrence of PCD in unicellular parasites has proved controversial because, whilst the morphologies observed are consistent with apoptosis, it appears that the pathways involved are different to those in mammalian cells where the majority of research has focussed [8,27].

It is likely that like with other eukaryote cells [28] various forms of programmed cell death may be important

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in protozoan parasites including autophagy [29]. The detection of, and semantics for, parasite apoptosis is the focus of other papers within this thematic issue (Jiménez-Ruiz *et al.*, 'Apoptotic markers in protozoan parasites'; Picot *et al.* 'Are protozoan metacaspases potential parasite killers?'). Here, we use the term 'apoptosis' to describe cells that have made the decision to die (analogous to suicide) as a strategy to improve transmission of surviving parasites. The distinction between apoptosis a strategy employed by parasites to die and apoptosis as simply the way in which parasites die when they are killed by host/vector factors is key; the former predicts apoptosis benefits all parasites in an infection, the latter predicts that a reduction in numbers is detrimental. As we are primarily interested in the evolutionary explanations for apoptosis to occur our focus is on apoptosis as a parasite strategy.

The occurrence of apoptosis in unicellular parasites is a challenge to explain because "Darwinian survival of the fittest" assumes organisms have evolved strategies to maximise their proliferation not their death. Here, we outline possible evolutionary explanations for apoptosis in protozoan parasites and suggest how they should be tested, with an emphasis on the importance of considering parasite ecology. We focus on malaria (*Plasmodium*) parasites as the application of an evolutionary framework to understand parasite life-history traits is better developed for malaria than other protozoan parasite species [30-33]. However, natural selection finds similar solutions to shared problems; therefore, it is likely that our message will be applicable more broadly to protozoan parasites. We start by outlining what is currently known about apoptosis in malaria parasites and the possible evolutionary explanations for why parasites would employ this strategy. We then go on to highlight the ecological factors which should be considered in choosing markers and conducting experiments on protozoan apoptosis, before suggesting possible future directions for testing the evolutionary explanations.

Apoptosis in malaria parasites

Whilst in their vertebrate host's circulation, *Plasmodium* parasites produce asexual stage parasites, which go through rounds of replication within their host's red blood cells, and so maintain the infection. They also produce sexually differentiated transmission stages (gametocytes) which no longer replicate but if taken up by the mosquito vector provide the potential for transmission [34,35]. When taken up in a vector's blood meal, gametocytes must immediately differentiate into male and female gametes and mate (Figure 1). Within 18-20 hours post fertilisation, each zygote transforms into a motile ookinete, which traverses the midgut wall and invades the epithelium of their vector. Here, each ookinete differentiates into an oocyst and divides asexually to produce thousands of sporozoites. When an oocyst ruptures, its sporozoites are released into the haemocoel to migrate to the salivary glands, ready to be injected into new hosts [34,36]. This whole process, termed sporogony, takes around 21 days for *P. berghei* in *Anopheles stephensi* [37].

Recent research has revealed that large numbers of ookinete stage parasites display a variety of apoptosis markers (e.g. condensed chromatin, fragmented DNA, caspase-like activity, translocation of phosphatidylserine and loss of mitochondrial membrane potential) (Table 1). This includes observations in the rodent malaria parasite *P. berghei* [24], as well as the human malaria parasite *P. falciparum* [25]. There is also evidence of apoptosis markers in zygotes of *P. berghei* [24] and in asexual stages of *P. falciparum* after treatment with the anti-malarial drug chloroquine [23,26,38] and a common apoptosis inducer staurosporine [38]. Our own data provide further evidence for apoptosis in the ookinete stage of *P. berghei* and also the first evidence for *P. yoelii* ookinetes (Table 1; Figure 2; Additional file 1). Furthermore, previously published accounts of *Plasmodium* parasites displaying 'crisis' or 'degenerate' forms may provide earlier examples of PCD in malaria [39-43]. Controlled experimental approaches have demonstrated that this phenomenon occurs independently of mosquito and host immune cells and is not unique to *Plasmodium* parasites; evidence for apoptosis across a range of protozoan parasites (including *Leishmania, Trypanosoma* and *Toxoplasma* spp.) is rapidly accumulating [18,19,22,44].

Protozoan parasites cause some of the most serious infectious diseases of humans, livestock, wildlife, and companion animals. The discovery that these unicellular organisms undergo apoptosis, and that the underlying molecular and cellular processes appear to differ from those of multicellular eukaryotes, offers a new paradigm for medical and veterinary interventions [23]. However, these differences have also resulted in controversy over which methods, terminology, and markers are appropriate for protozoan parasites. This debate must be resolved before parasite apoptosis can be understood: from the proximate genetic, molecular and cellular mechanisms that orchestrate cell death, to the ultimate evolutionary explanations for the existence of PCD in unicellular organisms.

Evolutionary explanations for apoptosis

For unicellular parasites, suicide may appear to be a counter-intuitive strategy when organisms are expected to have evolved to maximise their proliferation. However, uncontrolled replication often is not the best strategy for parasites as this may lead to the host or vector

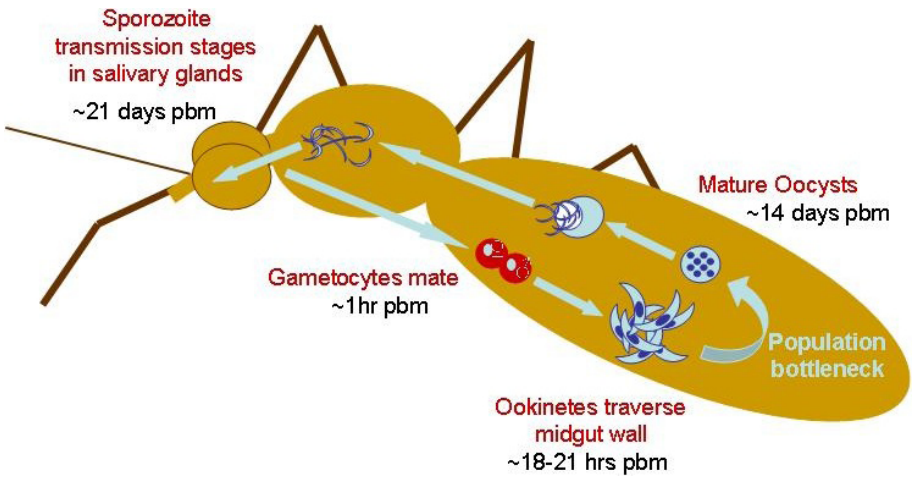

Figure 1 Life cycle of malaria parasites within the mosquito vector (sporogeny). Timings show approximate estimates of the stages post blood meal (pbm) for the progression of *P. berghei* though *Anopheles stephensi*. Apoptosis has been observed in three species of malaria parasites at the ookinete stage. The ookinete to oocyst transition is a well known bottleneck in the parasite life-cycle. Estimated reductions in numbers at this point in the life cycle are variable but for *P.berghei* in *A. stephensi* one estimate is a 35 to 120 fold reduction in parasite numbers depending on the density within the vector [37].

dying before there is a chance for transmission [45]. Evolutionary theory therefore suggests that under certain conditions parasites will be selected to display prudence in order to prevent premature death of their host, maximising the time and resources available for transmission and therefore their fitness [45-47]. Apoptosis in single celled organisms can be viewed as an extreme form of prudence and a cooperative (helping) behaviour.

It has been suggested that protozoan parasites may undergo apoptosis as a cooperative behaviour in order to prevent killing the host/vector [20,21,24,48,49]. However, evolutionary theory predicts that a cooperative behaviour will only evolve if the cost to the individual performing the behaviour (the actor) is outweighed by the benefit to the recipients weighted by the relatedness of the actor to the recipients [50,51]. A parasite committing suicide (via apoptosis) is an extreme form of cooperation, and obviously death is the highest cost payable. This means that the relatedness between apoptosing parasites and the survivors must be high, and that there must be a substantial benefit provided to the survivors, otherwise a trait as costly as suicide could not have evolved. If infections are initiated by one or very few clones then relatedness will be high. In this case, the question becomes 'how can overall transmission success (fitness) be improved by a reduction in parasite density?' For malaria parasites, there are at least two non-exclusive reasons why lowering parasite density could increase the chance of successfully completing the life cycle in the vector and being transmitted.

First, capping the density of ookinetes within a mosquito may prevent premature mortality of the vector by limiting damage caused either by ookinetes traversing the midgut epithelium or by later stages in sporogony [52]. Limiting damage to mosquitoes may be particularly important for malaria parasites as the development time required to be transmissible from the salivary glands (~3 weeks) is long compared to the average life expectancy of mosquitoes in the wild, which some estimates put at as low as 1-2 weeks for adult females of the *Anopheles* species [53]. Therefore, slight variations in the mortality rates of mosquitoes could have a significant effect on parasite transmission. The effect of malaria infection on mosquitoes is controversial with some studies finding a positive correlation between oocyst density and mosquito mortality, but others finding no evidence of a cost to lifespan [54]. These contrasting results may be due to artificially good conditions in lab experiments masking negative effects of malaria infection [54]. However, if parasites employ apoptosis to limit damage to mosquitoes then a benign effect of infection should not be surprising. Second, ookinetes at high density could directly effect sporozoite production if oocysts at high density compete for access to limited resources (e.g. nutrients), or indirectly by inducing stronger mosquito immune responses. Little is known about the developmental requirements of oocysts, but malaria infected mosquitoes are more likely to sugar-feed and divert resources away from reproduction (through apoptosis of their ovary cells) [55,56], suggesting that malaria infection causes a significant energetic burden.

Cooperation is widespread and recent advances in mathematical theory and empirical methods have revealed that the same general principles explain the evolution of cooperation and conflict across a wealth of

Table 1 Variation in rates of apoptosis and temporal patterns observed in malaria parasites

Species	Life cycle stage	ref	condition	Marker	Detection method	Proportion positive
P. berghei	ookinetes		*In vitro*	Condensed chromatin	Acridine orange (Sigma)	18 hrs - 15.5 (±1.06)%
						18 hrs - 34.5 (±1.76)%
		[83]	in PBS suspension			**22 hrs - 55.8 (±13.68)%**
						26 hrs - 49.01 (±5.51)%
				Fragmented DNA	TUNEL (histochemical, Calbiochem, UK)	**18 hrs - 48.55 (±6.01)%**
			or			**22 hrs - 64.19 (±6.09)%**
						26 hrs - 69.89 (±2.81)%
			In RPMI	Caspase-like activity	CaspaTag (Chemicon international, USA)	18 hrs - 17.0 (±2.12)
						18 hrs - 30.15 (±2.14)%
						22 hrs - 43.8 (±1.53)%
						18 hrs - 47.72(±3.93)%
				Translocation of phosphatidylserine	Annexin V- FITC apoptosis detection kit (Sigma, UK)	**18 hrs - 19.57 (±1.88)%**
						22 hrs - 28.33 (±5.61)%
						26 hrs - 30.12 (±2.75)%
				Mitochondrial membrane potential	JC-1 assay kit (Molecular Probes, UK)	18 hrs - 34.38 (±2.95)%
	ookinetes	[24]	*In vitro* **In RPMI**	Condensed chromatin	Acridine orange (Sigma)	**24 hrs - 31%**
						36 hrs - 80%
	ookinetes & zygotes mix		*In vivo*	Condensed chromatin	Acridine orange (Sigma)	18, 20 & 24 hrs - all over 60%
	ookinetes	[77]	*In vitro*	Translocation of phosphatidylserine	Annexin-FITC Apoptosis Detection Kit (Sigma, UK)	<3% (assay time not reported)
				Fragmented DNA	ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Chemicon International)	No positive cells observed (assay time not reported)
				Condensed chromatin	Acridine orange (Sigma)	No positive cells observed (assay time not reported)
				Caspase-like activity	CaspaTag (Chemicon international, USA)	**21 hrs - 3.8 (±0.05)%**
						24 hrs - 14 (±9.00)%
	ookinetes	*	*In vitro* **In RPMI**	Caspase-like activity	CaspaTag (Chemicon international, USA)	**15 hrs - 13.70 (±12.20)%**
						18 hrs - 13.06 (±6.42)%
						21 hrs - 45.90 (±11.00)%
						24 hrs - 67.94 (±4.83)%
	ookinetes		*In vitro* **In RPMI**	Fragmented DNA	*In situ* cell death detection kit, Flourescein (Roche)	**15 hrs - 9.38 (±4.44)%**
						18 hrs - 14.57 (±3.29)%
						21 hrs - 22.08(±8.96)%
						24 hrs - 9.24 (±3.09)%
	ookinetes	$	*In vitro* **In RPMI**	Caspase-like activity	CaspaTag (Chemicon international, USA)	**18 hrs - 20.06 (±3.50)%**
P. yoellii	ookinetes	*	*In vitro* **In RPMI**	Caspase-like activity	CaspaTag (Chemicon international, USA)	**15 hrs - No positive cells observed**
						18 hrs - 4.85 (±1.40)%
						21 hrs - 62.8 (±11.10)%
						24 hrs - 92.59 (±7.41)%

Table 1 Variation in rates of apoptosis and temporal patterns observed in malaria parasites *(Continued)*

	ookinetes		*In vitro* **In RPMI**	Fragmented DNA	*In situ* cell death detection kit, Flourescein (Roche)	**15 hrs - 7.29 (±3.84)%**
						18 hrs - 7.41 (±4.90)%
						21 hrs - 6.09 (±2.92)%
						24 hrs - 9.70 (±0.36)%
P. falciparum	ookinete	[25]	*In vivo*	Fragmented DNA	TUNEL (histochemical, Calbiochem, UK)	24 hrs - 67.8 (±2.82)%
	Asexual blood stages (trophozoites & schizonts)	[23]	*In vivo* after treatment with chloroquine	Loss of mitochondrial transmembrane potential	Carbocyanine dye JC-1	Timings and proportions positive not reported
				Fragmented DNA	TUNEL (fluorescent, Roche)	
		[26]	*In vivo* after treatment with chloroquine	DNA laddering	After electrophoresis, Southern blotting and autoradiography, a ladder pattern observed	Timings and proportions positive not reported
P. falciparum	Asexual blood stages	[38]	*In vivo* after treatment with chloroquine (CQ) or staurosporine (ST)	Loss of mitochondrial transmembrane potential	Cell-permeable lipophillic cation probe JC-1 (Molecular probes, Eugene, USA)	10% in untreated cultures increased to 31% (CQ) and 25% (ST)
				Caspase-like activity	CaspaTag (Chemicon international, USA)	10% in untreated cultures increased to 34% (CQ) and 32% (ST)
				Fragmented DNA	ApoDirect DNA fragmentation assay kit (Clontech, San Diego, USA)	10% in untreated cultures increased to 27% (CQ) and 56% (ST)

Results organised by life-cycle stage and study. Although the majority of studies summarised here have focused on the ookinete parasite stage, there is still considerable variation in the proportion of cells judged to be apoptotic. This variation may be due to differences in experimental set up between labs, e.g. the nutrients available to parasites and the densities of cultures. Assays of apoptosis may also vary at specific time points in the proportions of positive cells depending on the time scale of the processes being assayed. When parasites are assayed over a time course there is a general trend for an increase in positive cells with time. * Pollitt *et al.* reported here (figure 2) $ Pollitt *et al.* reported here (figures 4 & 5)

taxa (from bacteria to insects to humans). This framework predicts that ookinetes will undergo apoptosis when closely related parasites benefit, and ookinete numbers are high enough to negatively affect mosquito lifespan or sporozoite production. These predictions provide the specific, testable, hypotheses that: the proportion of apoptosing ookinetes will: (i) be density dependent and increase with the number of ookinetes in the midgut; and (ii) be greatest when ookinetes in an infection are clonally related and decrease as the genetic diversity of parasites sharing a vector increases. Examples of unicellular parasites cooperating with relatives in a density-dependent way are common [57,58]. Many of the best examples of this come from bacteria that form complex structures called biofilms to provide protection from the host immune response or antibiotic drugs [59], and bacteria that forage cooperatively to extract iron in a usable form from their host [60].

Despite the generality of the evolutionary principles that explain cooperation, the suggestion that apoptosis in malaria parasites is a social trait is controversial. Whilst in bacteria, quorum sensing mechanisms have been described to explain the coordination of behaviour [61], as yet, no specific quorum signalling system has been found for malaria parasites. However, evidence that malaria parasites respond to changes in their within-host environment by altering their resource allocation decisions show that they can detect and respond to factors such as the presence of competitors and variation in resource availability [31,62,63]. The predictions for why parasites undergo suicide are clear and testing them will resolve whether parasite apoptosis has been shaped by natural selection to enable parasites to cooperate with their kin. As with all emerging and interdisciplinary fields, undertaking the key, conceptually simple experiments required to test these predictions is constrained by the limitations of the methods and techniques available. For example in bacteria targeted disruptions have been useful in testing the fitness consequences of specific phenotypes (e.g. [64]), however, specific candidate genes for apoptosis in malaria are lacking and complex traits are difficult to disrupt. In the next section we outline the methodological constraints that currently impede the collection of data of high enough quality to undertake quantitative tests of the evolutionary explanations for parasite apoptosis. Given the medical and economic implications of malaria parasites and the drive to develop transmission-blocking intervention strategies, understanding their transmission biology from an evolutionary perspective is also timely and important.

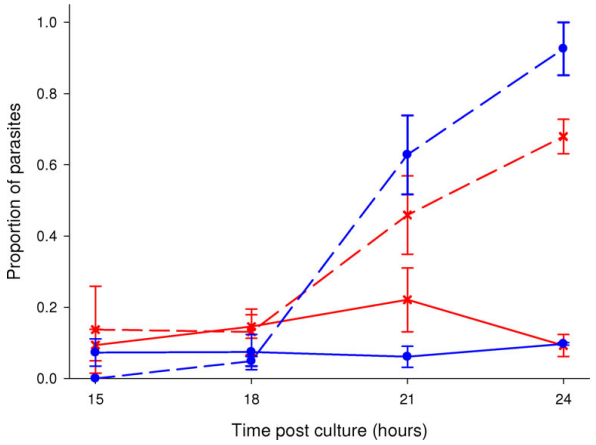

Figure 2 Markers for apoptosis vary over time and between species. Graphs show the proportion of ookinete stage parasites displaying DNA fragmentation as measured by TUNEL (solid lines) and Caspase-like activity (dashed lines) as measured by CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Fluorescein in conjunction with propidium iodide (Chemicon international, USA) in *Plasmodium berghei* (red lines with crosses) and *Plasmodium yoelii* (blue lines with circles). Bars show standard errors of the mean and values are based on between 3 and 6 individual infections per time point. For each mouse 8 ookinete cultures were set up which were incubated for 14 hours before the cultures from each mouse were pooled and purified for ookinetes using macs ls cell separation columns (Miltenyi biotec). The resulting purified ookinetes were then alliquated into 8 individual 1 ml cultures (1 per time point for 2 assays) containing complete ookinete media. These cultures were then returned to the incubator until the time relevant time point (15, 18, 21 or 24 hours post culture set up). More detailed methodology is available in Additional file 1.

Ecological considerations: applying assays for morphological markers

We suggest that to examine the evolutionary causes and consequences of apoptosis in malaria parasites, their ecology must be taken into account when deciding which assays to use and how best to apply them. In Table 2 we outline the markers and assays available, highlighting their suitability (from an ecological perspective) for use with malaria parasites, and we discuss the general issues below.

Which markers matter?

Many kits for assaying markers of apoptosis in mammalian cells are commercially available. However, it is not clear which markers are most suitable for measuring apoptosis in protozoan parasites. For example, one often used marker for apoptosis in mammalian cells is the translocation of phosphatidylserine to the outside of the cell membrane, which is detected using an annexin assay. In mammals phosphatidylserine provides a signal for the apoptotic cell to be engulfed by phagocytes, which prevents the cell from disintegrating and the resultant debris from causing inflammation. Whilst such a 'tidy death' is clearly advantageous in a muticellular organism, it may not be an applicable concern for single-celled ookinetes in the mosquito midgut. However, the presence of this marker in yeast and leishmania suggests that it may have additional functions [16,65]. In leishmania it is thought that phosphatidylserine translocation acts as a form of 'apoptotic mimicry' which aids the parasite in infecting macrophages [65]. In yeast the reason for the membrane altering during apoptosis is not yet known, however an interesting possibility is that it could act as a signal to other yeast cells.

Another common marker for apoptosis is caspase activity. In mammalian cells, classical apoptosis is triggered by the activation of caspases, which are apoptosis-specific cysteine proteases within the clan CD [66]. Part of the controversy surrounding the characterisation of parasite apoptosis stems from the absence of 'true' (canonical) caspases in protozoans [27,67]. However, ancient caspase homologues (known as metacaspases) are present in the genomes of plants, fungi and protozoa [68]. In plants and fungi metacaspases have been shown to be involved in apoptosis [69] and four metacaspases have been identified in the *Schistosoma mansoni* and *S. japonicum* [67,70] which could also play a role in a form of PCD. In *Plasmodium* there are three metacaspases (PxMC1, PxMC2 and PxMC3), and like the mammalian counterparts, they all posses a defined pro-region and a catalytic domain that is indicative of the clan CD, family C14 caspases. It has been suggested that one or more of these metacaspases can carry out a functionally analogous biological role to metazoan caspases, and although data are scarce, they have been linked to programmed cell death in some unicellular organisms [44,71-73]. Of the three metacaspases in *Plasmodium*, MC1 may represent the best candidate enzyme to be involved in a form of PCD in *Plasmodium* as it is the only MC that has the required predicted catalytic cysteine and histidine residues in the correct context for an active enzyme, and is typified by the *P. falciparum* enzyme PfMC1 (PF13_0289) ([74], Sajid personal communication) However, it is now evident, from work on mammalian cells, that beside the caspase dependent form of apoptosis a caspase independent form can also occur [8,75].

The role of metacaspases in parasite apoptosis is controversial, and contrasting conclusions have been drawn from different experiments [8,27,76]. Supporting data come from studies showing that the addition of the broad spectrum caspase inhibitor Z-VAD.fmk results in a reduction of the number of ookinetes displaying a variety of apoptotic morphologies, and also a doubling in the number of oocysts in the mosquito midgut [24]. In contrast, one study examining a *P. berghei* line in which PbMC1 has been deleted, found no loss of

Table 2 Summary of some commonly used markers for apoptosis

Marker of apoptosis	Example of assay used	Method of detection	Practical considerations	Relevance for malaria ecology
Activation of caspase-like molecules	CaspaTag (Chemicon international, USA)	A fluorescent labelled general caspase inhibitor (FAM.VAD.fmk (green) and SR.DEVD.fmk (red)) binds to active caspase within the cell. Positive cells fluoresce under fluorescent microscope	- Quick and easy to use	The role of caspase-like molecules is controversial in protozoan parasites, therefore it is not possible to be certain that apoptosis is being detected. However, if caspase molecules are a reliable marker they would be useful as an early marker of induction.
			-Results not as clear as with TUNEL	
			- Viability tests can be performed in conjunction	
			- Large scale experiments possible	
			The caspase inhibitor used in the caspase assay is broad spectrum and therefore may cross-react with unrelated molecules.	
Depolarisation of mitochondria outer membrane	JC-1 assay kit (Molecular Probes, UK)	JC-1 is a cationic carbocyanine dye that accumulates in mitochondria. Loss of mitochondrial membrane potential can be detected by the shifting of emission of fluorescence from orange (polarised mitochondrial membrane) to green (depolarised mitochondrial membrane).	- Quick and easy to use	The role of mitochondria in malaria apoptosis not well established. However, if markers prove to be reliable they would be useful as an early marker of induction.
			- Viability tests can be performed in conjunction	
Condensed chromatin	Acridine orange (Sigma)	Differentially stains SS and DS nucleic acids - enables the detection of condensed chromatin. Apoptotic cells should show an intense red staining in nucleus	- Quick and easy to use	Good relevance as we would expect this process to be the same for mammalian and protozoan cells.
			False positives possible and Results not as clear as with TUNEL	
			- Viability tests can be performed in conjunction	
			- Large scale experiments possible	
Translocation of phosphatidylserine to outer cell membrane	Annexin V-FITC apoptosis detection kit (Sigma, UK)	Positive display green annexin labelling on the cell surface, which can be detected by fluorescent microscopy	- Quick and easy to use	May not be relevant for malaria cells for two reasons.
			- Results not as clear as with TUNEL	1. The cell membrane of protozoan parasites is very different to that of mammalian cells.
			- Viability tests can be performed in conjunction	2. The ultimate reason for mammalian cells expressing phosphatidylserine on the outside of apoptotic bodies in order to be taken up by phagocytes, is not relevant for the mosquito midgut.
			- Large scale experiments possible	

Table 2 Summary of some commonly used markers for apoptosis *(Continued)*

Fragmented DNA leading to the generation of fragments with 3'OH groups	*In situ* cell death detection kit, Flourescein (Roche)	DNA of fixed and permeabilized cells labelled by the addition of flourescein dUTP at strand breaks by terminal transferase. Flourescein then detected by fluorescent microscopy (figure 3)	- More laborious than using Acridine orange, CaspaTag or Annexin V detection	Good relevance as we would expect this process to be the same for mammalian and protozoan cells. However as DNA fragmentation is thought to be a late process in apoptosis may only see markers at a later time point than induction of apoptosis pathways.
			- Gives clear unambiguous results.	
			- Requires cells to be dead so cannot perform viability tests in conjunction.	
			- Slides can be stored (at 5°C) for a few days allowing later analysis and therefore large scale experiments.	Some necrotic cells may show positive.
Morphological Markers e.g. membrane blebbing and formation of apoptotic bodies	Electron microscopy	Observation of cell morphology under electron microscope to detect membrane blebing or formation of apoptotic bodies	Time consuming and expensive	Malaria parasite cells differ in structural aspects from mammalian cells, it is therefore not clear whether the structural changes observed in mammalian cells would be relevant for these parasites. The ultimate reasons for formation of apoptotic bodies to be taken up by macrophages also not relevant in the mosquito midgut.
			- Not a good basis for morphological changes seen in malaria apoptosis	
			- Requires cells to be dead so cannot perform viability tests in conjunction.	
			- Large scale experiments not possible but may be useful in conjunction with other assays	
Detecting cell viability				
Propidium iodide (PI)	Propidium iodide (Roche)	Stain is taken up in cells with compromised membranes causing cells to display a red fluorescence.	- Quick and easy to use	Useful method for assessing viability of cells which can be used in conjunction with other assays of apoptosis.
			- Cells must be viewed quickly after application	
			- Can be used in conjunction with assays on live cells e.g. CaspaTag.	

Particular reference is paid to their ecological and practical considerations when using to test evolutionary predictions. For more details on the markers of apoptosis in protozoan parasites see paper by Jiménez-Ruiz *et al.* ('Apoptotic markers in protozoan parasites') in this thematic.

apoptosis (judged using CaspaTag) and therefore concluded that it may be a functionally redundant gene [77]. However, these authors did not apply any other markers to their knock out line and found very low rates of phosphatidylserine translocation and no DNA fragmentation or chromatin condensation with their wild-type line. Our own research has shown that when compared to genetically intact parasites, this PbMC1 knock out line reaches higher ookinete and oocyst densities in infected mosquitoes and that this translates into lower sporozoite production and higher mosquito mortality (Pollitt *et al.*, unpublished data). This demonstrates there is a cost to sporogony at high parasite densities and suggests that PbMC1 mediates ookinete numbers although it is not yet clear if this is through apoptosis.

Recent research has implicated clan CA cysteine proteases in chloroquine mediated apoptosis [38]. However, the use of inhibitor/probes developed for use in humans or other systems with canonical caspases should be viewed with caution. To date there are no proteases from Plasmodium that have a specificity for Asp at P1 (as per Val-Ala-Asp (P3-P2-P1)) of the caspase selective probes. This together with irreversible nature (fmk) of

these probes is likely to lead to off target inhibition and the clan CA may be amongst these off target hits [78]. This problem is compounded if these inhibitors are used either at very high concentrations or they are used over an extended time period.

How should assays be applied?

One of the attractions of using the activation of caspase-like molecules as a marker for apoptosis is the ability to assay cells in the early stages of apoptosis. This is important for studies aiming to assay apoptosis at biologically relevant time points. For example, ookinetes begin to develop from retort (immature) forms at around 8 hours post fertilisation and begin to invade the gut epithelium at 18-20 hours for *P. yoelii*, although these timings vary between malaria parasite species [36]. If some ookinetes undergo apoptosis in order to provide a benefit to others, we expect apoptosis will be initiated before 18-20 hours post fertilisation. Therefore, it is important to assay apoptosis rates before ookinetes have either died or transformed to the next life cycle stage. Assays for depolarisation of mitochondrial membrane potential may also have the advantage of being an early marker of apoptosis [79,80], however, as with many of assays discussed here the relevance for malaria parasite cells has not yet been established. Intuitively, assaying the activation of death executors would appear to be the best approach when testing whether levels of apoptosis are linked to developmental schedule, but early apoptotic mammalian cells can be saved [81] and it is not yet known at what stage parasites become irreversibly committed to dying.

A possible solution to this problem is to assay morphologies observed at the end of the apoptosis program, such as DNA fragmentation. However, this approach may complicate the ambition of examining apoptosis in a biologically relevant timeframe. The key question is how long does the apoptosis program in malaria parasites take? For example, if ookinetes are predicted to initiate apoptosis at 18 hours post fertilisation, how much later should DNA fragmentation be assayed? We show a preliminary examination of this issue in Figure 3. The proportion of cells with fragmented DNA at the time points measured are significantly lower than those displaying caspase-like activity, but clearly more studies are required to characterise the time lags between the activation of apoptosis and the appearance of the resulting morphologies. These studies should be designed with reference to the developmental schedules of parasite species that undergo sporogony at different temperatures. Another problem with assaying late-stage apoptosis morphologies is the validity of combining assays to distinguish between apoptotic cells and necrotic cells. Furthermore, any temporal variation in how individual parasites initiate and progress through apoptosis will make it difficult to distinguish between cells in a sample that are healthy, undergoing apoptosis, have died by apoptosis, or have died by necrosis. This could be further complicated by the fact that assays such as TUNEL for DNA fragmentation can also be positive in necrotic cells. This all points to the importance of having reliable time-lines for the changes associated with the different forms of cell death.

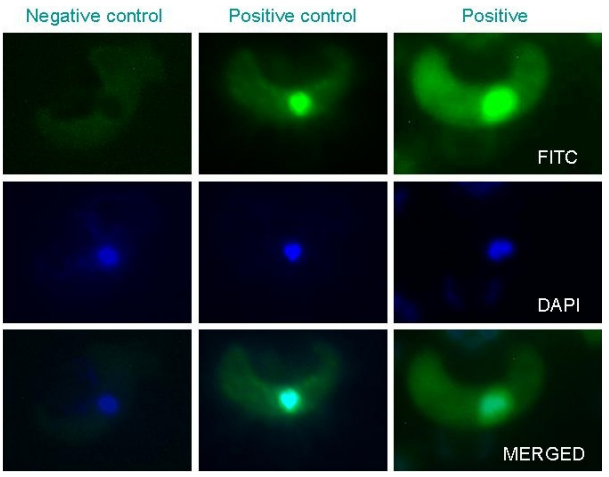

Figure 3 Identification of DNA fragmentation by fluorescent TUNEL assay. Images of *P. berghei* ookinetes with fragmented DNA marked using a TUNEL assay (*In situ* cell death detection kit, Fluorescein, Roche) at 21 hours post culture. Positive cells show a bright green nucleus. Negative controls were incubated with only the label solution without the enzyme and positive controls had DNA strand breaks induced with DNase 1 recombinant (3 units/ml for 10 mins at 20°C) before labelling (DNase 1 recombinant, grade 1, Roche). DAPI staining was used to check the location of the nucleus. More detailed methodology is available in Additional file 1.

In our experience, measuring DNA fragmentation by fluorescent TUNEL assay appears to provide repeatable and non-subjective results (positive cells show an obviously fluorescing nucleus; Figure 3). Fragmentation of DNA is also a well defined end point to a program of programmed cell death. However, because TUNEL assays are applied to fixed and permeabilized cells, parasites that do not display DNA fragmentation cannot be further characterised as healthy or necrotic. For this reason, assays that can be applied to live parasites are very useful, such as CaspaTag (but see 'which markers matter' section). Because most apoptosis assays have been developed for mammalian cells, the protocols involved may not always be appropriate for live parasites. For example, ookinete stage malaria parasites develop at considerably lower temperatures and would experience significant stress and heat shock if treated at 37°C during assaying. Electron microscopy on parasite nuclei can be useful to verify and compare processes in order to reliably discriminate between apoptosis and other forms

of programmed cell death. However, this is not practical for hypothesis testing on large numbers of cells. For most experiments wanting to study ecological variation then it is also necessary that assays allow high throughput of samples. In these situations assays on dead and fixed parasites that can be stored for later analysis (e.g. fluorescent TUNEL) are more practical.

In addition to studies that characterise parasite apoptosis programs, technical developments are required so that assays can be applied to large numbers of cells and enable their morphologies to be efficiently and accurately quantified.

Variation: noise or not?

A major challenge for evolutionary biology is explaining variation in traits observed across genotypes, and within the same genotype in different environments. The relative proportions of parasites recorded as undergoing apoptosis varies over time, between markers and across studies (see Table 1). This variation is common in evolutionary studies of phenotypic traits and initially seems difficult to interpret. The challenge is to identify patterns and understand what is driving them. Recent studies have revealed that malaria parasites detect and respond to subtle changes in the conditions they encounter during infections by altering traits such as investment in gametocytes and their sex ratio [31,62,63,82]. These conditions include the density of clone-mates and genetic diversity of co-infecting parasites, which are also the factors predicted to influence levels of apoptosis. Therefore, variation across studies in infections and/or experimental set up may result in differences in the cues that parasites experience or their ability to detect this information.

Our data show that even within controlled replicate infections - initiated with the same infective dose of the same parasite clone in the same batch of hosts - there is variation in the proportion of parasites displaying markers of apoptosis (Figure 4). This may be due to parasites responding to subtle variation in parasite densities or other aspects of their within-host environment, such as immune challenge or anaemia. However, when the data from individual infections within an experimental group are combined and we compare two replicate experimental groups, we see that individual replicates are noisy but patterns are consistent (Figure 5). This suggests that it is possible to reliably detect patterns but large sample sizes (number of independent infections) and standard conditions are required. Also, where possible, variables such as parasite density should be recorded in order to control for its potential influence on rates of apoptosis.

Parasite ecology may also be important for understanding variation in apoptosis behaviour between different parasite species. For example, a comparison of our data for rates of apoptosis observed in *P. berghei* and the related rodent malaria species *P. yoelii* (Figure 2) reveals significantly different patterns of caspase-like expression over time (species*time χ^2_1 = 4.38, p = 0.046). The proportion of parasites assaying positive

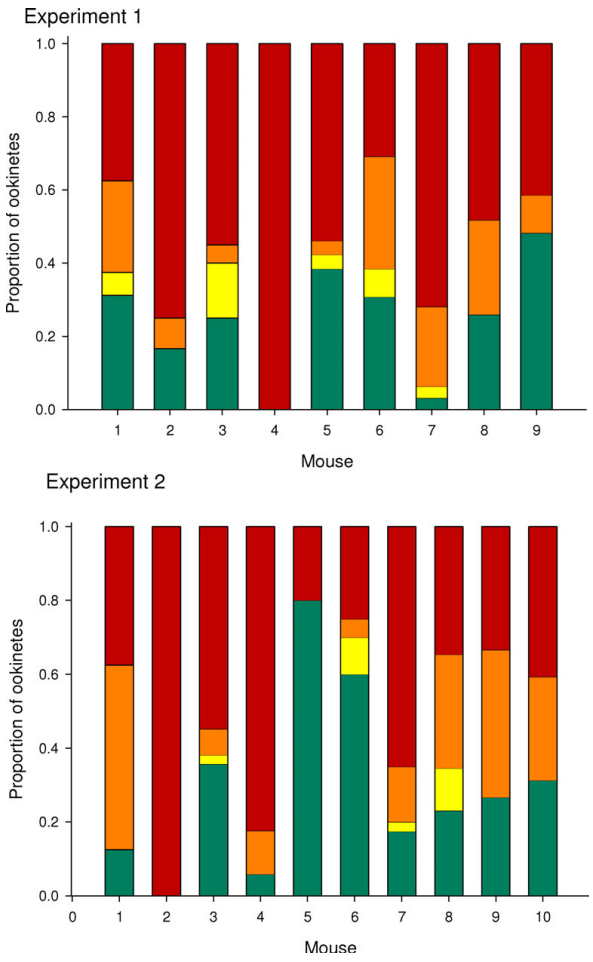

Figure 4 Replicate experimental infections show variation for proportions of parasites showing caspase-like activity and viability. Two sets of replicate experiments were set up by infecting 10 male MF1 mice 8-10 weeks old per replicate with 10^7 *P. berghei* parasites after pre-treatment with Phenylhydrazine 2 days pre-infection (120 mg/kg). One mouse from experiment 1 failed to become infected so was removed from the study. Cultures were then set up 4 days post infection. After 18 hours ookinetes were purified using MACS LS cell separation columns (Miltenyi biotec) and a minimum of 30 parasites were assayed for caspase like activity and viability using CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Fluorescein in conjunction with propidium iodide (Chemicon international, USA). Green indicates healthy ookinetes negative for caspase-like activity with intact membranes, yellow indicates early apoptotic ookinetes displaying caspase-like activity with intact membranes, orange indicates late apoptotic ookinetes displaying caspase-like activity but also compromised membranes and red indicates dead cells with compromised membranes [25]. More detailed methodology is available in Additional file 1.

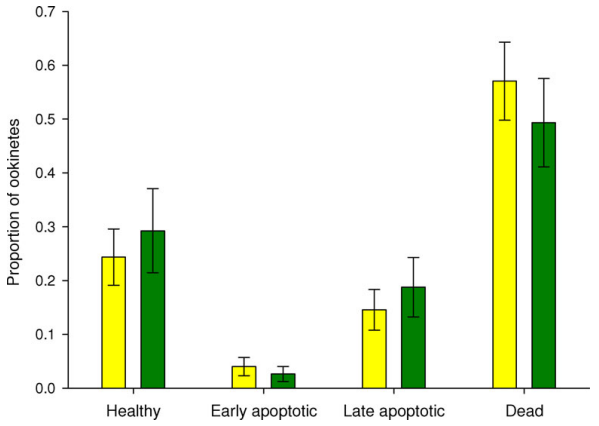

Figure 5 Between experiments markers of apoptosis and death are repeatable. Data taken from the experiments described in figure 4. Average proportion of ookinetes classified into 4 categories (healthy, early apoptotic, late apoptotic or dead) across 9 replicate infections for experiment 1 (yellow) and 10 replicate infections for experiment 2 (green). Although there is variation for replicate infections within experiments (figure 4) there is no significant variation between the cells categorised into each condition between 2 experiments carried out on different days (χ^2 = 5.81 (3 df), p > 0.1). Error bars show the standard error of the mean.

with TUNEL was not significantly different over time (χ^2_1 = 0.001, p > 0.5). However the proportion positive at 21 hours was significantly lower in *P. yoelii* (t = 2.37, df = 8, p = 0.045). Given the debate over the reliability of using caspase-like activity as a marker of apoptosis in *Plasmodium*, we propose that a more accurate picture is gained from focussing on the TUNEL positive cells. Can differences in species ecology explain the difference in levels of DNA fragmentation in these species? A possible explanation is that as *P. berghei* parasites can reach considerably greater oocyst densities than *P. yoelii*, higher rates of apoptosis are required for *P. berghei* survivors to gain a benefit in terms of increased transmission success. With this in mind it would be interesting to examine the rates of apoptosis in other malaria parasite species.

Conclusions

The discovery of apoptosis-like cell death in single celled protozoans such as malaria parasites provides an exciting challenge for evolutionary biology to explain and a new direction for intervention strategies. Progress in both of these fields requires evolutionary biologists to work together with cell biologists to develop reliable high throughput assays to study variation in apoptosis in response to the key parameters of parasite density and infection genetic diversity. At the same time, debates on the best markers for assaying apoptosis and appropriate terminology need to be resolved.

Additional material

Additional file 1: Supplementary information. More detailed Methods for our data presented here can be found in the supplementary information.

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Authors' contributions

LCP collected the results presented here and wrote the first draft of the manuscript, SER assisted in the design of experiments and helped to draft the manuscript. NC, SKM, MS all participated in the formation of the final version of the manuscript and helped to draft specific sections. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

Materials and methods

Infections and culturing parasites

We used the rodent malaria parasites *P. berghei* 820 and *P.yoelii* wild type from the WHO Registry of Standard Malaria Parasites (The University of Edinburgh). Infections were initiated in male MF1 mice (8-10 weeks old) which had been pre-treated with Phenylhydrazine (PHZ) (60mg/kg (3 days prior to infection) or 120mg/kg (4 days prior to infection) for *P.yoelii* and *P.berghei* infections respectively). This treatment stimulates the erythropoietic response in the mice encouraging the establishment of the malaria infection and the production of gametocytes [83]. Infections with both species were initiated with 10*7 parasites and infected blood was collected for culturing on day 3 or 4 post infection for *P.yoelii* and *P.berghei* respectively. Ookinete cultures were set up with 75µl of infected blood in 5mls of complete ookinete culture media (RPMI + 10% fetal calf serum, pH 8) and incubated at 24°C or 21°C respectively for *P.yoelii* and *P.berghei*. After incubation ookinete cultures were purified using MACS LS cell separation columns (Miltenyi biotec).

Detection of Caspase-like activity and cell viability

Caspase-like activity was measured using CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Fluorescein iodide (Chemicon international, USA) following the manufacturers instructions except for modification of incubation temperature which were changed from 37°C to 24°C or 21°C respectively for *P.yoelii* and *P.berghei* cells. Previous studies have shown that lowering the incubation temperature to a suitable level for ookinete development does not alter the proportion of cells showing caspase-like activities and that higher temperatures result in increased death in ookinetes as measured by membrane viability [25]. CaspaTag utilises a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase (FAM-VAD-FMK) which acts as a non-cytotoxic fluorescent broad-spectrum caspase inhibitor that binds covalently to active caspases in living cells. After incubation cells which contain the bound reagent (indicating caspase-like activity) will glow green when analysed by fluorescence microscopy.

Fragmented DNA

DNA fragmentation was measured using TUNEL *In situ* cell death detection kit, Fluorescein (Roche) following the manufacturers instructions. Purified ookinete cultures were centrifuged, smeared and fixed onto a glass slide with 4% Paraformaldehyde in PBS (pH 7.4, 1 hour incubation at 15-21°C). Cells were then permeabilised using 0.1% Triton X-100 in 0.1% sodium citrate (2 minutes on ice). Fixed permeabilised cells where then incubated with the TUNEL reaction mixture which labels DNA strand breaks, by terminal deoxynucleotidyl transferee (TdT), which catalyzes polymerization of labelled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL-reaction). Flourescien labels incorporated into the nucleotide polymers are can then detected by fluorescence microscopy (the nucleus of positive cells fluoresce bright green, see figure 3). After incubation with the tunnel reaction mixture VECTASHIELD mounting medium with DAPI (vector laboratories) was added to samples to prevent the fluorescence fading and allow the location of the nucleus to be confirmed.

Cell viability

Cell viability was measured using propidium iodide (PI) (250µM/ml) (Chemicon international, USA) which enters cell with permeabilised membranes resulting in red fluorescence.

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ECOLOGICAL IMMUNOLOGY

Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology

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Summary

1. Ecoimmunologists aim to understand the costs, benefits, and net fitness consequences of different strategies for immune defense.

2. Measuring the fitness consequences of immune responses is difficult, partly because of complex relationships between host fitness and the within-host density of parasites and immunological cells or molecules. In particular, neither the strongest immune responses nor the lowest parasite densities necessarily maximize host fitness.

3. Here, we propose that ecoimmunologists should routinely endeavour to measure three intertwined parameters: host fitness, parasite density, and relevant immune responses. We further propose that analyses of relationships among these traits would benefit from the statistical machinery used for analyses of phenotypic plasticity and/or methods that are robust to the bi-directional causation inherent in host-parasite relationships. For example, analyses of how host fitness depends upon parasite density, which is an evolutionary ecological definition of tolerance, would benefit from these more robust methods.

4. Together, these steps promote rigorous quantification of the fitness consequences of immune responses. Such quantification is essential if ecoimmunologists are to decipher causes of immune polymorphism in nature and predict trajectories of natural selection on immune defense.

Key-words: bivariate statistics, *Daphnia*, evolutionary parasitology, immunocompetence, optimal immunity, random regression, resistance, tolerance

Introduction

Hosts vary greatly in the strength of their immune responses and their capacity to defend themselves against parasites. Ecoimmunologists shed light on this variation by characterizing optimal defense strategies in a world of life-history tradeoffs, unpredictable epidemics, polyparasitism, and genetic and environmental variation (Medley 2002; Rolff & Siva-Jothy 2003; Lazzaro & Little 2009; Sadd & Schmid-Hempel 2009). Accordingly, a basic requirement of empirical studies in ecoimmunology is to measure and interpret the fitness consequences of immune responses – in other words, to ascertain the impact of cellular or molecular responses to infection (hereafter, 'immune responses') upon the lifetime reproductive success (hereafter, 'fitness') of the responder. But this basic requirement poses serious challenges.

Ecoimmunologists increasingly appreciate that two 'shortcuts' to estimating the fitness consequences of immune responses must be avoided. The first is to count immunological cells or molecules and assume that hosts producing the most hemocytes or antibodies, for example, are the most fit (e.g. Nunn, Gittleman & Antonovics 2000 as critiqued by Read & Allen 2000). The second is to quantify parasite densities and assume that hosts bearing the most parasites are the least fit (e.g. see critique in Behnke, Barnard & Wakelin 1992). These shortcuts fail because the magnitude of an immune response does not always correlate positively with host fitness

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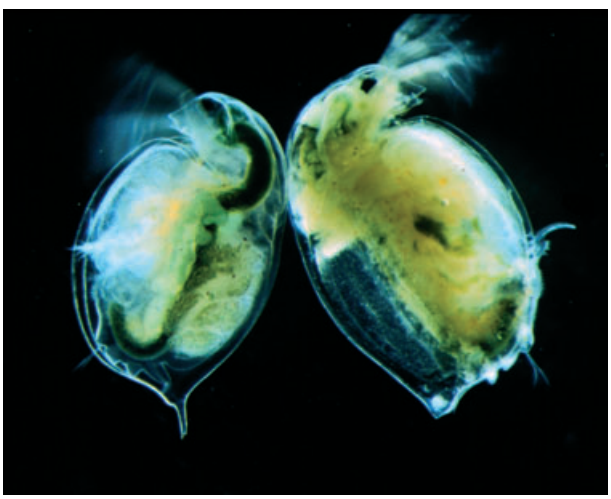
(Adamo 2004; Graham, Allen & Read 2005; Rolff & Siva-Jothy 2003, Sadd & Schmid-Hempel 2009, Viney, Riley & Buchanan 2005), and hosts that kill all of their parasites are not necessarily better off: host fitness may be maximal at some intermediate parasite density (Behnke, Barnard & Wakelin 1992; Viney, Riley & Buchanan 2005; Stjernman, Raberg & Nilsson 2008). As a result, the relationship between host fitness and parasite density – sometimes called tolerance by evolutionary ecologists – has received a lot of attention lately (Raberg, Sim & Read 2007; Ayres & Schneider 2008, 2009; Pagan, Alonso-Blanco & Garcia-Arenal 2009; Raberg, Graham & Read 2009); also see summary of controversy below.

Here, we aim to cement the view that ecoimmunologists should aim to quantify how host fitness is affected by both parasite density and immune response magnitude. Measuring this triad of traits offers the best opportunity to interpret ecological variation in immunity. We stress that each trait is likely to be the product of an interplay between host and parasite genes, which has important consequences for empirical practice and for inferring evolutionary outcomes. We propose that a combination of controlled experiments and statistical

Box 1. From evolutionary genetics to ecoimmunology in lab and field: *Daphnia magna–Pasteuria ramosa* as a 'model' system

Daphnia are small (~1–3 mm), ubiquitous freshwater crustaceans that have been the focus of a large and diverse literature, including toxicology, life-history, physiology, nutrition and parasitology. *Daphnia* were also the subject of pioneering work on invertebrate cellular immunology (Metchnikoff 1884), an area that has recently been revisited within the ecoimmunology framework (Auld, Scholefield & Little 2010) (Boxes 2 and 3). In the field, gathering epidemiological data is relatively straightforward because the clear carapace of *Daphnia* makes many infections easy to identify. In the photograph, the left *D. magna* is healthy (note embryos in the brood chamber), while the right *D. magna* is infected with the bacterium *Pasteuria ramosa,* which sterilizes hosts leading to an empty brood chamber (a clear indication of reduced host fitness). Epidemics are common and severe in this system, but highly variable in space and time (Stirnadel & Ebert 1997; Duncan, Mitchell & Little 2006; Lass & Ebert 2006; Duncan & Little 2007). With parasite density and indeed parasite fitness being further quantifiable because transmission spores are easily counted, the recommended triad of traits – host fitness, within-host parasite density, and immune response magnitude – are measurable.

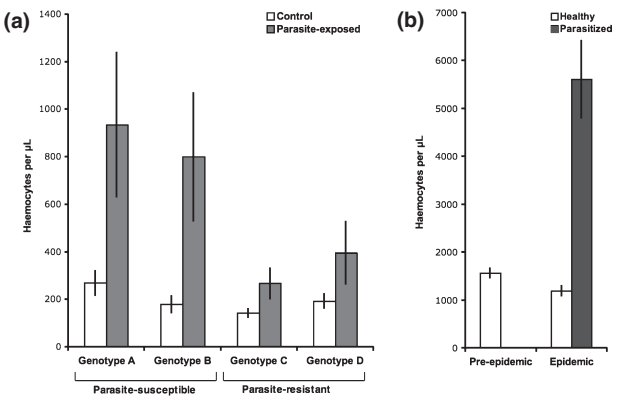


Adding power to these studies is the possibility to gain insight into genetic effects through controlled experimentation. Especially important for this experimentation is the fact that *Daphnia* are facultative parthenogens and can be cloned, which enables precise comparison of genetic backgrounds, or the study of different environments on replicates of the same genetic background. Experiments on susceptibility of *D. magna* to *P. ramosa* have revealed extensive genetic variation in both hosts and parasites (Ebert, Zschokke-Rohringer & Carius 1998; Little & Ebert 1999, 2000, 2001), including genetic specificity – that is, host genotype by parasite genotype interactions where the susceptibility of a host genotype is tightly dependent on the parasite strain to which it is exposed (Carius, Little & Ebert 2001). Similar 'context-dependence' has been revealed when hosts and parasites have been studied under different environmental conditions (genotype by environment interactions; Vale & Little 2009; Vale, Stjernman & Little 2008). Furthermore, the short generation time of *Daphnia* (~ 10 days) enables the study of real-time evolutionary responses to parasites (Little & Ebert 1999, Duncan & Little 2007, Zbinden, Haag & Ebert 2008). The *Daphnia* system is also unique for the accessibility of reconstruction of historical genetic changes via the resurrection of resting stages (Limburg & Weider 2002; Decaestecker *et al.* 2007).

Box 2. An immune measure for the dead

The study of a putative immune response in the crustacean *Daphnia* provides a simple yet striking example of the dangers of assuming that a stronger immune response represents greater host fitness. Immune responsiveness in *Daphnia* can be estimated by extracting a small amount of hemolymph and counting the abundant plasmatocytes (cells that appear to have phagocytic function). Different genotypes of *D. magna* show markedly different susceptibilities (Carius, Little & Ebert 2001) to the naturally coevolving bacterial pathogen *P. ramosa,* and recent work has revealed that immune responses are evident only in susceptible genotypes (see panel A below; Auld, Scholefield & Little 2010).

These data are from an experiment involving four long-term laboratory *Daphnia* lines for which resistance characteristics are well-established. Two lines are highly susceptible and two are highly resistant, and replicate hosts from each line were either exposed or not exposed (controls) to a spore suspension of *P. ramosa*. Compared to their controls, the susceptible genotypes showed a substantial increase in the number of circulating phagocytes in an 8-h period of exposure (data are a mean of six replicates studied from four time points: 2, 4, 6, and 8 h of exposure). An expanded data set on sixteen host genotypes largely confirmed this pattern (See Auld, Scholefield & Little 2010). Thus, *D. magna* may have a two-stage defence – a genetically determined barrier to parasite establishment, and a cellular response once establishment has begun. A strong immune response is a marker for susceptibility rather than resistance.



This result has since been borne out in field studies comparing hemocyte counts in naturally infected and uninfected hosts. Many *D. magna* populations experience summer epidemics of *P. ramosa,* and it can be shown that pre-epidemic hosts (which are of course not infected) have low hemocyte counts, comparable to healthy hosts during the epidemic period (panel B, above). The pre-epidemic samples represent a mean from three sampling dates in May, 2009, whilst the epidemic samples represent a mean of 13 sampling dates spread from June to October 2009 when *P. ramosa* was common in the population; S. K. J. R. Auld, A. L. Graham & T. J. Little, unpublished). *Pasteuria ramosa* sterilizes its host, and so hosts showing signs of infection (and thus high cell counts) will not directly contribute genes to the next generation. Thus, in the *D. magna–P. ramosa* interaction, a strong immune response is not associated with high fitness, but rather is tightly linked to being genetically dead.

methodologies borrowed from other branches of biology can disentangle relationships among the three traits. Our statistical advice is focused on rigorous exploration of relationships between host fitness and parasite density (i.e. evolutionary ecological tolerance).

WHEN MORE IS BLATANTLY NOT MORE: AN EXAMPLE

We begin by illustrating the benefits of three-trait data sets with an example, the crustacean *Daphnia magna* infected with the bacterium *Pasteuria ramosa* (Box 1). Several decades of both laboratory and field research have generated a deep understanding of the fitness consequences of parasitism in *D. magna* (Ebert 2005). Consequently, unlike ecoimmunological work in which hemocyte or white blood cell densities, for example, are quantified without knowledge of host fitness or relevant parasite biology, ecoimmunology of *D. magna* can be undertaken with extensive knowledge of potential evolutionary outcomes. Different host genotypes show markedly different susceptibilities to infection (Carius, Little & Ebert 2001), and yet after exposure, densities of responding hemocytes are highest in susceptible genotypes (Auld, Scholefield & Little 2010) (Box 2). Had hemocyte densities been measured in *D. magna* hosts without either prior knowledge of the system or knowledge of the infection status of individuals – that is, without the understanding that cellular responses are a marker for both genetic susceptibility and infection – we might have naively concluded that hosts with highest hemocyte densities would have the highest fitness. However, hosts with the most hemocytes actually tend to have the lowest fitness because they're infected with a sterilizing parasite! This example strikingly demonstrates that more is not necessarily more

Table 1. An array of ecoimmunological study designs which may be experimental or observational, performed in the field, the laboratory, or both

Design	Description	Possible measurements	Examples
1	Experimental: induce immune response to non-infectious agents in the field or lab	Host fitness *Immune response* *Density of natural parasites*	a, b, c
2	Experimental: infect with different doses of parasites, primarily in the lab	Host fitness *Immune response* *Parasite density*	d, e, f, g, h, i
3	Experimental: infect with different parasite genotypes, primarily in the lab	Host fitness *Immune response* *Parasite density*	j, k, l
4	Experimental: remove parasites in the field or lab	Host fitness *Immune response* Parasite density	m, n
5	Observational studies in the field	Host fitness Immune response Density of natural parasites	o, p

We argue that nearly any design would benefit from inclusion of immune response and parasite density measurements, to accompany measurement of host fitness. Possible measurements in plain text are not optional; items in *italics* are optional but recommended (see text). [a]Bonneaud *et al*. (2003), [b]Moret & Schmid-Hempel (2000), [c]Raberg & Stjernman (2003), [d]Ben-Ami, Ebert & Regoes (2010), [e]Bleay *et al*. (2007), [f]Lundgren & Thorpe (1966a), [g]Lundgren, Thorpe & Haskell (1966b), [h]Nol, Olsen & Rhyan (2009), [i]Xiao *et al*. (2005), [j]Carius, Little & Ebert (2001), [k]Grech, Watt & Read (2006), [l]Raberg, Sim & Read (2007), [m]Hudson, Dobson & Newborn (1998), [n]Pedersen & Greives (2008), [o]Norris, Anwar & Read (1994), [p]Stjernman, Raberg & Nilsson (2008).

in immunology, that well-studied host-parasite systems may be poised to make major contributions to ecoimmunology, and that host fitness and parasite densities [or other readouts of the efficacy of defense (Adamo 2004; Viney, Riley & Buchanan 2005)] must be measured alongside immune responses.

Three key traits in the context of ecoimmunological study designs

Various study designs enable ecoimmunologists to quantify the fitness consequences of immune responses (Table 1). Here, we highlight the role that the three focal measurements (host fitness, immune response magnitude, and parasite density) can play in each, to emphasize that more measurements per study rather than radically new study designs will go a long way to improving empirical ecoimmunology. We illustrate with examples, but have not attempted to be exhaustive.

We make several qualifications from the outset. First, fitness in terms of lifetime reproductive success is not easy to measure, but it remains an aspiration. Proxies such as annual survival, annual fecundity, or health must have demonstrated relevance to true fitness for the system under study. Secondly, the appropriate immunological and parasitological measurement(s) will vary greatly from system to system. We discuss how to promote selection of relevant parameters below. Thirdly, when quantification of parasite density is impossible but longitudinal studies are feasible (for instance, studies undertaken on free-ranging animal populations in the wild), duration of infection (e.g. days parasite positive) might in principle serve as the parasitological readout, though we know of no such studies to date. Fourthly, we caution that multiple independently-derived stocks of the parasite or immunostimulant may be needed, depending on the level of generalization desired. For instance, if only one strain of *Plasmodium* was used in an experiment [or indeed in years of experiments, as frequently observed in laboratory infection models (Viney 2006)], it is difficult to generalize to the fitness consequences of malaria as these may differ dramatically across strains/species. Finally, field and laboratory research have different weaknesses. In particular, field studies may be confounded by unknown exposure histories of hosts, whereas lab studies often use both host and parasite strains of restricted genetic diversity (Viney 2006). We believe that the most powerful ecoimmunological studies will combine such data (e.g. Box 2) and would encourage development of more systems that span the field-lab divide while quantifying host fitness, parasite density, and immune response magnitude.

DESIGN 1: EXPERIMENTS IN THE ABSENCE OF INFECTION

A common ecoimmunological study design involves non-infectious experimental manipulations such as injection with agents that spark immune responses [e.g. lipopolysaccharide (LPS) or vaccines; Design 1 in Table 1]. For example, injection of LPS into house sparrows followed by fitness measurements demonstrated that reproductive costs of immune responses may be compensated for by greater investment in the next clutch, among other mechanisms (Bonneaud *et al*. 2003). Injection of LPS into bumblebees demonstrated that survival costs of immune responses might only be expressed when resources are limited (Moret & Schmid-Hempel 2000). A key advantage of using parasite mimics rather than true infections is avoidance of the confounding influence of the mechanisms the parasite uses to circumvent immune responses (Huxham, Lackie & Mccorkindale 1989; Barnes & Siva-Jothy 2000).

Studies of Design 1 can be enriched by measurement of cellular or molecular immune responses. A particularly good example is the study of blue tits injected with tetanus-diphtheria vaccine, in which survival was monitored and vaccine-specific antibodies measured; a major finding was stabilizing selection on primary antibody responses to diphtheria (Raberg & Stjernman 2003). In other words, birds with either very weak or very strong responses to that antigen were unlikely to survive the winter. The birds probably do not experience diphtheria. Instead, the titre of vaccine-induced antibodies might be considered an index of overall immune responsiveness: weak responders are presumably prone to infectious diseases in general, hence their high mortality rate, while the high mortality rate of strong responders might arise from general or vaccine-induced costs of immunity (Raberg & Stjernman 2003).

For any study of Design 1, a difficulty is that the relevance of the induced response to an animal's ability to fight a real infection is rarely known (Adamo 2004; Staszewski & Boulinier 2004; Viney, Riley & Buchanan 2005; Martin, Weil & Nelson 2006). For example, does the magnitude of response to LPS predict responsiveness to live bacteria? Similar questions arise for the assumed relationship between diphtheria-specific antibodies and resistance to real infections of the blue tits described above. In principle, studies of Design 1 can be broadened to include measurement of the within-host densities of relevant parasites. This enables researchers to address whether strong responses to immunostimulants are correlated with lower prevalence or intensity of real infections (e.g. Lee *et al*. 2006). Indeed, we support calls for studies of Design 1 to provide 'functional readouts' (Viney, Riley & Buchanan 2005) or 'host resistance tests' (Adamo 2004) that lend insight into the ability of hosts to fight real infections.

DESIGNS 2–4: EXPERIMENTS IN WHICH INFECTIONS ARE ADDED OR REMOVED

The fitness consequences of strong immune responses probably depend upon the number and genotype of parasites with which a host is infected. Ecoimmunological experiments in which infections are added to or removed from hosts (Designs 2–4) aim to test that hypothesis. Just as data on immune response magnitude and/or parasite density make Design 1 studies more informative, the same applies to these designs.

Design 2, in which hosts are challenged with varying doses of live parasites, is commonplace in biomedical research, with the dose at which 50% of hosts can no longer prevent infection (infectious dose, ID_{50}) or survive infection (lethal dose, LD_{50}) serving as indices of host susceptibility. Indeed, dose-response experiments can reveal whether completely resistant host genotypes exist and, more generally, quantify the distribution of host susceptibility in a population (Ben-Ami, Ebert & Regoes 2010). When accompanied by immunological measurements, such experiments can also demonstrate whether there is a threshold number of parasites above which immune elements are induced, qualitatively altered, or else overcome (Bleay *et al*. 2007). If hosts die above a particular inoculating dose despite controlling parasite numbers, then disease may be due to a cytokine storm (uncontrolled production of signalling molecules, particularly by the innate immune system) or other immunopathology (Graham, Allen & Read 2005). A virulence factor of methicillin-resistant *Staphylococcus aureus* (MRSA) exhibits such dose-dependence: at low doses it induces protective innate immune responses, while at high doses it induces septic shock (Yoong & Pier 2010). The severity of other infections may entail similar dose-dependent shifts to immunopathology (e.g. among microparasites of vertebrate hosts (Schmid-Hempel & Frank 2007)). Such patterns have even been observed in invertebrates. In *D. magna*, for example, very high spore doses of *P. ramosa* may lead to drastic reductions in host fitness, even though parasite density often decreases with increasing dose (Ebert, Zschokke-Rohringer & Carius 2000). The benefits and costs of strong immune responses can therefore be obscured in studies of Design 2 unless parasite density and/or immune response magnitude are also measured as experimental outcomes.

Design 3, in which the experimenter varies the parasite genotype or species to which hosts are exposed, is indispensable for identification of genetic specificity of attack and defense that underpins so much of co-evolutionary theory (e.g. Carius, Little & Ebert 2001; Grech, Watt & Read 2006). Again, parasite density and immunological measurements aid interpretation by providing some mechanistic detail of within-host events. For example, whether the sickest hosts bear high parasite densities, cytokine storms, or both, can be shaped by parasite genotype (Long *et al*. 2008) and lead to different evolutionary trajectories (Day, Graham & Read 2007).

Design 3, accompanied by parasite density measurements, was used in the first declared test for tolerance in animals (Raberg, Sim & Read 2007). The study demonstrated that host genetic background conditioned how fitness (i.e. health of laboratory mice, in this case anaemia and cachexia) changed with increasing malaria parasite density. Mouse strains that experienced the shallowest declines in fitness with increasing parasite density were considered the most tolerant (Raberg, Sim & Read 2007). However, interpretational problems arise when parasite diversity and density are confounded – more generally, when density is not experimentally controlled – or when tolerance mechanisms are unknown, as discussed in detail below.

For a variety of ethical and logistical reasons, both Designs 2 and 3 may be difficult to apply outside the laboratory. For example, one may (rightly) be forbidden to infect wild animals experimentally. A possible exception would be to add ecoimmunological analysis onto epidemiological susceptibility studies such as those used to assess the potential for wild hosts to sustain transmission of zoonotic infections such as rickettsia, brucellosis, or monkeypox (Lundgren & Thorpe 1966a; Lundgren, Thorpe & Haskell 1966b; Xiao *et al*. 2005; Nol, Olsen & Rhyan 2009).

Better yet, Design 4, in which parasites are experimentally removed from wild hosts, is likely to be informative and

applicable across a wide variety of systems. Such experiments have been used to quantify how parasites (particularly nematodes) regulate host population size (Hudson, Dobson & Newborn 1998; Pedersen & Greives 2008), but the experiments can also reveal costs of parasitism borne by individuals and, in principle, the costs and benefits of immune responses (Pedersen 2005, Pedersen & Greives 2008). For example, following clearance of nematodes, measurements of the density of other parasites and the magnitude of subsequent immune responses can disentangle mechanisms of within-host interaction, as has been advocated for observational studies (Bradley & Jackson 2008). Design 4 seems a rich vein for future experimentation in ecoimmunology.

DESIGN 5: ECOIMMUNOLOGICAL OBSERVATIONS

When fitness measurements are coupled with data on parasite densities and/or immune response magnitude, purely observational studies can also yield rich insights (Norris, Anwar & Read 1994; Stjernman, Raberg & Nilsson 2008). For example, blue tits with both very low and very high densities of Apicomplexan parasites exhibit reduced overwinter survival (Stjernman, Raberg & Nilsson 2008). The data suggest that strong immune responses themselves are associated with mortality risk, while weak immune responses increase risk of mortality due to infection. Such an inference would be supported by evidence that birds with the lowest parasite densities exhibit the strongest parasite-specific immune responses. To our knowledge, such a data set does not yet exist, though the data of Raberg & Stjernman (2003) on vaccine-specific antibody and survival of blue tits (discussed above) lend support. Another observational ecoimmunological study – of the Soay sheep of St. Kilda – gains tremendous power via longitudinal tracking of survival, fecundity, and lifelong parasite densities of individual sheep (Clutton-Brock & Pemberton 2004). Immunological measurements have now demonstrated an association between antibody titres and the ability of sheep to resist nematodes (Coltman *et al.* 2001) and to survive harsh winters (Graham *et al.* in press).

One problem with observational studies is that a wild host that bears few parasites might not necessarily be resistant to infection, but might instead have avoided exposure (Sheldon & Verhulst 1996). It is sometimes possible to pair observational data with experiments that distinguish these distinct causes of parasite density – for example, in the case of potential environmental influences on both exposure and susceptibility of amphibians to trematode infections (Rohr *et al.* 2008) or dose-response experiments on *D. magna* (Ben-Ami, Ebert & Regoes 2010). However, when controlled experiments are impossible, immune response measurements can also help to distinguish whether exposure or resistance best explains low parasite density (Bradley & Jackson 2008). For example, if helminth-free hosts bore high titres of IgE, then the inference of resistance to infection would be supported (Bradley & Jackson 2008).

WHICH PARASITES AND IMMUNE RESPONSES TO MEASURE?

The examples above highlight the value of measuring parasite density and/or immune response magnitude in the context of most ecoimmunology study designs, to 'open the black box' of mechanisms operating within hosts. For study systems that are not yet well characterized, exactly what to measure may not be obvious – for example, if the entire parasite fauna of the focal host species is unknown, or if the type of immune response required to kill a particular parasite is difficult to extract from the encyclopaedia of immunological possibilities. We suggest that opening the black box enough to permit evolutionary ecological inference does not require hugely specialized knowledge of parasitology and immunology. It does require dedication, however, and a willingness to think beyond LPS, phytohemagglutinin (PHA), sheep red blood cells (sRBC), and other tried and true but nonetheless limited workhorses of ecoimmunology (Adamo 2004; Viney, Riley & Buchanan 2005; Martin, Weil & Nelson 2006).

Of course, the final decision of what to measure hinges on both relevance and feasibility. Relevant parasites are likely to be the most prevalent/abundant in the environment or in hosts, though they might also be parasites that are rare but cause severe disease (Grenfell & Dobson 1995). The over 130 years of publications in parasitology and infection biology may provide excellent clues on what parasite(s) to measure, especially if related host species have received attention. Feasible parasites are those for whom samples can be obtained, ideally noninvasively, and for whom density (or at least prevalence) can be quantified. Blood and faeces are good places to begin looking for parasites of vertebrates (or invertebrates; e.g. Lazzaro, Sackton & Clark 2006), and for parasites such as helminths and protozoa, little more than vital stains and basic microscopes might be required. PCR-based techniques can make the detection of parasites feasible from almost any tissue.

The relevant immune response to measure often follows on from the relevant parasites, because the immune system to a large extent must tailor parasite killing mechanisms to the size, location (intracellular vs. extracellular, as well as gut vs. blood vs. other anatomical location), and route of entry of parasites (Schmid-Hempel 2005; Weaver & Murphy 2007). Thus, for example, if nematodes are prevalent and deadly, as among the Soay sheep, then it makes sense to target nematode-specific IgA for measurement (Clutton-Brock & Pemberton 2004). If instead blood-borne Apicomplexans are prevalent and deadly, as among Hawaiian birds, then it would be better to measure malaria-specific cytophilic IgY (Lee *et al.* 2006). Targeted measurement of cellular responses in *D. magna* (Box 2) and other invertebrates makes sense because many innate immune responses are based primarily on phagocytic cells (e.g. Elrod-Erickson, Mishra & Schneider (2000). These cells also generate non-specific reactive oxygen and nitrogen species or phenoloxidase that destroy pathogens and can

also be measured (Rolff & Siva-Jothy 2003; Rivero 2006). In vertebrates, it can be also be informative to measure non-specific molecules such as complement or natural antibody (Adamo 2004). Feasibility for immunological measurements is determined by the availability or development of appropriate tools for each host species (Bradley & Jackson 2008). We do not underestimate the difficulty of this enterprise (Matson *et al*. 2006), but we also feel that the benefits of working with real parasites and real immune responses (see also Martin, Weil & Nelson 2006) cannot be overstated.

Relationships among traits

Of course, choosing the right parasites and immune responses to measure is just one step. Next, the causal relationships among traits must be considered. This issue was highlighted at the beginning of this article with the *Daphnia* example, where a large immune response indicates susceptibility. The general point is that an immune response of a particular magnitude can either be a cause OR a consequence of a particular parasite density. For example, a high antigen-specific antibody titre can be indicative of resistance to infection by parasites bearing that antigen, but it can also indicate persistence of that antigen in the host.

Measuring both parasites and relevant immune responses is key to resolving directionality, because a negative correlation between them is predicted if immune responses cause resistance, whereas a positive correlation is predicted if immune responses merely reflect antigen load or present parasite density (see also Sheldon & Verhulst 1996; Lee *et al*. 2006, Whiteman *et al*. 2006; Bradley & Jackson 2008). If the magnitude of an appropriate effector immune response is uncorrelated with parasite density, then tolerance may be at work. That said, the magnitude and even the sign of these relationships can change over the course of infection. For example, early in infection, as immune responses ramp up, there may be a positive association between parasite densities and concentrations of immunological molecules. Later in infection, once most parasites have been cleared, the correlation may become negative. Controlled laboratory experiments will be critical to clarify these dynamics. Manipulative experiments in which immunological tools like monoclonal antibodies are used to alter levels of effector activity (e.g. Long *et al*. 2008) can reveal the extent to which particular immunological cells or molecules control parasite density in some systems. Longitudinal field studies – for example, of the dynamics of *Borrelia* exposure and *Borrelia*-specific antibodies in seabirds (Staszewski *et al*. 2007) – may also be informative. Indeed, theoretical groundwork for exploring relationships between parasite density and immune response magnitude has been laid, but data are lacking (Fenton & Perkins 2010).

Another key relationship in our triad of recommended traits is that between parasite density and host fitness. In the rest of this section, we outline analytical problems inherent in the study of this relationship and propose statistical solutions that should apply equally to relationships among all traits in the triad.

DEFINING TOLERANCE

Evolutionary ecologists have come to call the relationship between host fitness and parasite density tolerance (Raberg, Sim & Read 2007; Ayres & Schneider 2008, Ayres & Schneider 2009, Pagan, Alonso-Blanco & Garcia-Arenal 2009; Raberg, Graham & Read 2009). We note that this differs from the definition of tolerance in vertebrate immunology – that is, a lack of responsiveness to antigen that is actively maintained by cells of the immune system and essential to avoiding autoimmunity, for example (Abbas *et al*. 2004). However, we also note that cellular tolerance of parasite antigens can lead to organismal tolerance of parasites (Mills 2004), so the verbal definitions are not entirely at odds. The quantitative definition of tolerance poses greater challenges.

Tolerance according to the evolutionary ecological definition is the ability of hosts to limit the fitness costs of a given parasite density, but the quantitative definition has varied. In some theoretical (e.g. Roy & Kirchner (2000) and empirical (e.g. Ayres & Schneider 2008) studies, tolerance has been considered at a single parasite density, where two host genotypes bear the same number of parasites, but one genotype achieves higher fitness and is thus more tolerant of a given parasite density ['point tolerance' (Little *et al*. 2010)]. In other studies, tolerance has been considered a slope, quantifying how host fitness declines with increasing parasite density; more tolerant genotypes lose fitness less quickly as densities increase ['range tolerance' (Little *et al*. 2010)]. Genetic variation for range tolerance of rodent malaria was studied by Raberg, Sim & Read (2007), using an approach in line with studies of tolerance to herbivory (Tiffin & Rausher 1999; Simms 2000), though in plant studies the focus has been fitness (e.g. seed set) per unit of direct and measurable *damage* (e.g. leaf area lost due to herbivore chewing), while animal studies have thus far focused on fitness per parasite (see Baucom & de Roode in this issue). What is worrying is that alternative quantitative definitions – that is, point vs. range tolerance – can generate contradictory conclusions. For example, for two host genotypes that differ in range tolerance, their reaction norms will cross at some point in the range of parasite densities. If tolerance is estimated from relative fitness at a single parasite density, then the conclusion of which genotype is most tolerant depends upon where in the density range the underlying reaction norms cross, and the density at which point tolerance measurements are made (discussed in detail in Little *et al*. 2010).

Whenever possible (e.g. via dose-response experiments) range tolerance seems preferable to point tolerance to provide more comprehensive information about the fitness consequences of different defense strategies. However, range tolerance also raises complex analytical issues familiar to evolutionary biologists who study traits shaped by phenotypic plasticity or co-evolution.

HOW FITNESS DEPENDS ON PARASITE DENSITY: TOLERANCE AS PLASTICITY

If fitness is measured across a range of parasite densities, then range tolerance is directly analogous to the concept of plasticity under a linear reaction norm model (Scheiner 1993). It therefore seems likely that recent methodological advances in modelling phenotypic plasticity might usefully be applied to studies of tolerance. For example, fitness (W) of host genotype i at parasite density D might be modelled as:

$$W_{iD} = \mu + d.D + g_i + e \quad \text{Model 1}$$

where μ is the overall mean fitness, d is the average regression of fitness on parasite density (i.e. the mean range tolerance), g_i is the effect (relative to the overall mean) of having genotype i, and e is a residual error. In practical terms, this model could be parameterized as a linear mixed effect model with g_i fitted as a random effect. This would allow estimation of the variance in g_i, which is properly interpreted as an estimate of the genetic variance for host fitness (under a parasite challenge) in the population from which tested host genotypes were drawn. However, Model 1 is only appropriate if the host genotypes differ in their average fitness (i.e. there is among-genotype variance in g_i) and not in the slopes of their regressions on parasite density. When this holds, estimates of point tolerance will yield the same fitness ranking of host genotypes regardless of the value of D at which they are tested [i.e. the reaction norms do not cross (Little *et al.* 2010)].

Alternatively, g_i may itself depend on D if range tolerances differ between genotypes. Assuming that a linear model of this dependence of g_i on D is appropriate we should then expand our model such that:

$$W_{iD} = \mu + d.D + g_{\text{int}.i} + g_{\text{slope}.i}.\ D + e \quad \text{Model 2}$$

where $g_{\text{int}.i}$ is a genotype-specific effect on mean host fitness (relative to μ) while $g_{\text{slope}.i}$ is a genotype-specific effect on the regression of host fitness on parasite density. This model could be parameterized by adding a genotype by parasite density term to the random effect structure of the mixed model in a random regression (so-called because the regression is contained with the random effect structure of the model). This approach is increasingly being used to model reaction norms across environmental gradients (Nussey, Wilson & Brommer 2007). On a practical note, it is often useful to zero-centre the D axis such that the estimate of variance in g_{int} can be interpreted as the genetic variance for fitness under an average parasite density (i.e. when $D = 0$).

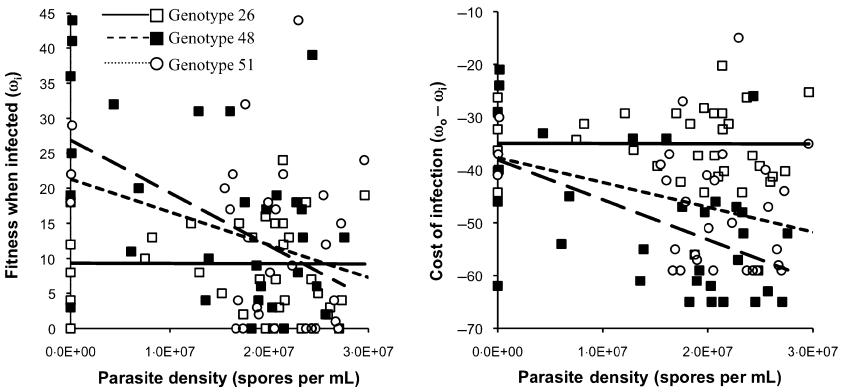
However, the key point to take from Model 2 is that, as outlined verbally by Little *et al.* (2010), if genotypes differ in their reaction norm slopes (i.e. there is variance in g_{slope}) then we expect the relative fitness ranking of different genotypes to change with D (though not necessarily within the range of parasite densities tested, nor within a biologically relevant range). A second point to note is that by formulating Model 2 as a mixed effect model a researcher can – and should – explicitly account for the covariance between reaction norm slopes and intercepts. Failure to account for this covariance can generate biologically misleading results because the information needed for evolutionary inference will often be influenced by the way in which tolerance relates to fitness in the absence of infection (the intercept). Host genotypes will almost certainly show fitness differences in the absence of infection – that is, genetically determined life-history variation is common (Stearns 1992). These differences may be linked to variation in the traits that contribute to defense via pleiotropy, as follows. One scenario is where defense against parasites is traded-off against vigor – that is, where a host possessing an allele that confers more potent defense is less fit than other genotypes when parasites are not around. But even in the absence of trade-offs, measurement of fitness of both infected and uninfected hosts is key, and *a priori* omission of intercepts from analyses of range tolerance (e.g. Raberg, Sim & Read 2007) may greatly limit inference about evolutionary outcomes. In Box 3, we illustrate this using data from the *D. magna–P. ramosa* system. Measuring the intercept of the reaction norm should be routine in laboratory studies of tolerance in which it is feasible to include control animals that are unexposed to the focal infection.

HOW FITNESS DEPENDS ON PARASITE DENSITY: CAUSATION AND CO-EVOLUTION

Another concern about the study of range tolerance in animals centres on the issue of causation. This is because parasite density, host fitness, and even immune responses are likely to be under the joint control of the host and the parasite. For example, leaving aside environmental effects on exposure, parasite density within a host is the result of the parasite's intrinsic replication rate and the host's ability to kill parasites. Immune response magnitude is the result of the host's intrinsic responsiveness and the immunogenicity of, or immunosuppression by, the parasite. Finally, host fitness when infected depends on all of the above, plus parasite virulence, plus host tolerance (Little *et al.* 2010)! Parasite growth within hosts is therefore difficult to experimentally control [even when controlling for genotype-by-genotype or genotype-by-environment interactions (e.g. Box 1)]. This problem may not apply to macroparasites such as helminths that do not replicate within the host (Bleay *et al.* 2007) or that have resting stages (Stopper *et al.* 2002), and thus their densities can be largely controlled via inoculating dose, but the problem certainly pervades the study of microparasites. Consequently, microparasite density at time t can be considered an uncontrolled outcome of the experiment, as opposed to an explanatory variable in the sense of regression or analysis of covariance (Sokal & Rohlf 1995). Here, it is not possible to disentangle whether parasite density determines host health (and by extension, host fitness), or if host health determines parasite density: they fundamentally confound each other.

Box 3. Inferring evolution from linear relationships between parasite density and host fitness

Many empirical studies have considered the linear relationship between parasite density (within hosts) and a measure of host fitness. Although a linear relationship may not always be representative, it can be adequate over some ranges of parasite density. But even in these cases, there are nuances to consider, in particular regarding the role played by host fitness in the absence of infection, that is, the *y*-intercept. Perhaps the majority of studies on the relationship between parasite density and host fitness have sought to gain insight into parasite evolution (evolution of virulence studies; e.g. De Roode *et al*. 2005), and thus the measurement of host traits in the absence of infection has been understandably ignored. Similarly, tolerance studies might not consider fitness in the absence of infection (which we call ω_0) because tolerance, by definition, does not include ω_0. And yet, it is difficult to make inference about selection on tolerance when ω_0 is not measured. First, the fitness of a particular genotype will be determined by both ω_0 and its fitness across parasite densities. These two components of fitness may not be independent due to pleiotropic effects, but even when they are, jointly considering how they covary sheds light on what the rank fitnesses of different genotypes might be. Secondly, it may not be realistic to estimate ω_0 from a *y*-intercept of a parasite density-host fitness relationship in a study that has not directly measured ω_0.



To highlight these points, we present the results of an experiment that exposed the crustacean *D. magna* to the bacteria *P. ramosa* (see Box 1 and Appendix S1, Supporting information). Fifteen replicates of each of twelve host genotypes were exposed to the parasite, and the number of offspring produced by infected hosts was counted. Later, infected hosts were killed and the density of parasite transmission spores (per mL of host tissue) was estimated. Thus, we gained the data necessary to plot parasite density (within-hosts) against host fitness (in this case measures of fecundity). For convenience, we use ω_i to represent the 'fitness of infected hosts'. We also measured the reproductive output of control hosts, that is, the fitness of those hosts not exposed to the parasite, ω_0. Full experimental details are presented in Supporting information.

We studied the relationship between parasite density and host fitness in two ways. First, we studied only 'fitness of infected hosts', ω_i. Secondly, we incorporated host fitness in the absence of infection (ω_0), by studying simply $\omega_0 - \omega_i$. As ω_0 represents what hosts can achieve in the absence of infection, $\omega_0 - \omega_i$. is the cost of infection. The two graphs above compare fitness when infected (ω_i) and the cost of infection ($\omega_0 - \omega_i$) across parasite densities.

For clarity, results for only three of the 12 genotypes are depicted, and we multiplied the cost of infection by (−)1 so that higher values represent greater fitness, making the two graphs visually comparable. Of particular note here is how inference regarding which is the most fit genotype changes depending on the fitness measure used. When examining only ω_i (fitness when infected), left graph, the genotype (26) that is the most tolerant in terms of range tolerance (i.e. shows the flattest slope) is less fit than the less range tolerant genotypes, except at the very highest parasite densities. However, in looking at the cost of infection, that is, once the response variable incorporates information about fitness in the absence of infection ($\omega_0 - \omega_i$, right graph), the most tolerant genotype is also potentially the most fit. The other two genotypes also switch their rank order of fitness over most, but not all parasite densities. The reason for these differences is that ω_0 is not accurately estimated by the relationship between parasite density and ω_i. Indeed, including all 12 host genotypes, a correlation between the *y*-axis intercept, as estimated from linear functions such as those in the left graph, shows no relationship with the actually measured fitness in the absence of infection ω_0 (spearman $\rho = -0{\cdot}2028$, $P = 0{\cdot}51$). How the cost of infection will ultimately determine the winner of a competition between genotypes will be determined by the local frequency of epidemics.

Thus, host genetic variation for range tolerance represents how genotypes differ in the strength of a relationship (typically studied as a regression) between parasite density and health/fitness, but it is difficult to say why. This becomes pertinent when considering the process of natural selection: without understanding the cause of differences in the

strength of relationships, it is not clear what trait is being selected upon and what evolutionary response to selection we should expect to see. For instance, it is possible that molecular mechanisms of tolerance control the relationship. If, for example, an immunological mechanism [e.g. anti-toxin or anti-inflammatory molecules (Raberg, Graham & Read 2009)] can be shown to alleviate disease severity as parasite numbers increase, it becomes more straightforward to interpret how natural selection will act on variation in range tolerance. This is because the immunological mechanism might then be understood to be the trait subject to natural selection. In the absence of such a mechanism, however, it is equally possible that different genotypes are just more or less sensitive to the laboratory environment, leading to differences in health and then parasite load. Here, we run the risk of confounding tolerance of the environment with tolerance of the infection. Interpreting the relationship between parasite density and fitness requires considerable caution because it is explicitly the product of two interdependent measures.

Similar issues have been discussed in other fields, and seem dangerous to ignore. For example, Ridley (1988), in his treatment of the benefits of multiple mating in insects, contrasted 'experimental comparisons' (with controlled explanatory variables), with 'non-experimental comparisons' (the uncontrolled, descriptive approach). In the latter kind of study, the risk is that experimental individuals in a sense self-select which treatments groups (once mated, twice mated, etc.) they are in, perhaps due to their condition. This self-selection may seem justified if randomly allocating individuals to treatments beforehand (the correct approach) entails significant loss of experimental subjects if some proportion of replicates fail to complete the required number of matings. However, it has become clear that different conclusions have been drawn about insect mating behaviour depending on the method used (Ridley 1988); see also Torres-Vila, Rodriguez-Molina & Jennions (2004). The similarities to experimental infection studies are obvious, as hosts (and parasites) may 'self-select' how a given dose turns into a given parasite density. Although this imposes a constraint on experimental design and inference, it cannot be ignored.

With respect to the study of tolerance, we gain some traction on the problem by applying a range of parasite doses, although (as outlined above) dose will often show complex relationships with microparasite density – for instance it may be highly nonlinear [e.g. *Pasteuria* in *Daphnia* (Ebert, Zschokke-Rohringer & Carius 2000)], or dose may influence the timing but not the magnitude of peak parasite density [e.g. *Plasmodium* in *Mus* (Timms *et al*. 2001)]. Alternatively, it may be feasible to inoculate with a single parasite dose and then apply a range of subcurative doses of an anti-parasite drug, although we know of no examples of this approach in which tolerance was quantified and we would caution that various potential confounding effects, especially if the drug has a direct impact on host health or if initial dose is all that matters, require careful thought. Injection of LPS or heat-killed bacteria might be informative for quantifying tolerance of septic shock. Lastly, there is the potential to use a range of parasite genotypes that differ in the density they tend to reach (Raberg, Sim & Read 2007), although this tendency would have to be independent of host genotype – that is, host genotype by parasite genotype interactions (*sensu*; Carius, Little & Ebert 2001) would confound this approach. Overall, statistical approaches that can account for the interdependency of measures in ecoimmunological data sets seem warranted.

BEYOND REGRESSION-BASED APPROACHES

In our discussion of phenotypic plasticity, we highlighted ways in which statistical methods such as random regression might benefit ecoimmunology. However, our advocacy of such methods should not distract from the fact that important, but largely unrecognized, statistical issues arise when neither experimental control of parasite density nor investigation into mechanism are feasible. First, if parasite density is not experimentally controlled it will necessarily be measured with error that is typically unaccounted for in regression based analyses of tolerance. Under simple (type I) linear regression, measurement error in the explanatory variable will lead to underestimation of the magnitude of the slope (i.e. overestimate tolerance) (Sokal & Rohlf 1995). This problem could be avoided by use of type II or major axis regression. However, a second issue is that any regression model specifies and assumes a uni-directional cause-effect relationship between parasite density (the independent variable) and host fitness (the response). As outlined above, however, there are good biological reasons to expect that the relationship to be bi-directional. Statistical models must always make simplifying assumptions and we do not suggest that regression be abandoned, only that violated assumptions be more widely recognized and that alternative, complementary types of analyses warrant consideration. For instance, while correlation can never prove causation, path analysis and structural equation modelling might allow different models of causal relationships between the measured host and parasite processes to be considered (and in some cases statistically compared) (Mitchell 1992; Shipley 1997).

Alternatively, there is considerable logic in choosing to treat both parasite density and host fitness as response variables in a bivariate analysis. For instance, using a bivariate mixed model (Lynch & Walsh 1998), the observed covariance between parasite density (D) and host fitness (W) can be modelled and decomposed into components attributable to factors of biological interest (e.g. host genotype or source population) and experimental design (e.g. block). For example, by fitting host genotype as a random effect (and assuming that repeated observations on each genotype are available) the total variance (V) in a trait (x) can be decomposed into a portion attributable to host genotype and a residual component (attributable to unmodelled environmental effects and measurement error). In a bivariate model the total variance–covariance matrix for two traits can be similarly partitioned such that:

$$\mathbf{P} = \mathbf{G} + \mathbf{R}$$

where **P** is the phenotypic variance–covariance matrix between n (in this case 2) traits, **R** is the matrix of residuals (usually interpreted as environmental effects), and **G** is the genetic covariance matrix

$$\mathbf{G} = \begin{bmatrix} V_{G(W)} & \mathrm{COV}_{G(WD)} \\ \mathrm{COV}_{G(WD)} & V_{G(D)} \end{bmatrix}$$

where $V_{G(W)}$ and $V_{G(D)}$ are the among-host genotype (i.e. genetic) variances for fitness and parasite density, respectively, while $\mathrm{COV}_{G(WD)}$ is the genetic covariance term. If so desired these parameters could be rescaled to yield the heritabilities of W and D (seen as traits of the host) as well as the genetic correlation, although it should be noted that these will typically be broad-sense (as opposed to additive) genetic parameters if clonal replicates are used. Moreover, these models are not limited to the study of genetic correlations, and they are not limited to bivariate. Researchers could include all response variables in a single model, and can then extract almost any pairwise linear relationships, including regressions, that are of interest.

This approach also provides an unexploited link to quantitative genetic models of trait evolution, since the genetic covariance between a trait and (relative) fitness actually provides an unbiased prediction of the expected selection response (Robertson 1966; Morrissey, Kruuk and Wilson, in press). A simple corollary of this is that even if there is an association between host fitness and parasite density, evolution of the host mechanisms for controlling the parasite density is not expected if $\mathrm{COV}_{G(WB)} = 0$ and all covariance arises from environmental sources of covariance (portioned into **R**). Given suitable data, further partitioning of **P** is readily achieved by addition of further random effects. While additional random effects may certainly be used to test specific hypothesized sources of environmental covariance between D and W (e.g. maternal effects, host cage effects), a second genetic covariance structure may be estimated in the event that multiple parasite genotypes were used (with replicate observations for each). Thus, it is possible to model W and D as traits that vary, and covary, as a consequence of interacting host and parasite genotypes, and to estimate the relative contributions of each to observed (co)variance. In this way genetic control of W and D need not be assumed to lie with either the host or the parasite, but rather can be influenced by both. We encourage ecoimmunologists to explore these approaches in more detail across a range of organisms.

Optimal studies of optimal immunity

With this article, we suggest three primary improvements to the empirical framework for ecoimmunology. In brief, we urge researchers to make more measurements, to choose them wisely, and to analyse them using some of the statistical techniques that have permeated other fields and are recommended above. The additional measurements (immune response magnitude and parasite density, to complement host fitness in the context of various study designs; Table 1) help to dissect important details of within-host dynamics – for example, are hosts more likely to die of high parasite densities or of immunopathology (Graham, Allen & Read 2005)? Wise choice of which immune elements and parasites to measure ensures relevance to fitness but requires basic knowledge of the infection biology of the target hosts or of related, well-investigated model systems (Bradley & Jackson 2008). Finally, statistical methods used in other branches of evolutionary biology appear more appropriate than current methods for dealing with inherent issues in ecoimmunological data sets (e.g. bi-directional causal relationships). We provide preliminary statistical advice for studying tolerance, but the suggested methods should apply to any data on host fitness, parasite density and/or immune responses. Together, our suggestions promote robust quantification and interpretation of fitness consequences of immune responses. We hope to prompt researchers to tailor suggestions according to what is most reasonable and appropriate for their systems and research goals. Most studies are imperfect (including those of the authors), but with steps such as those explored here, studies of ecoimmunology and optimal immunity (Viney, Riley & Buchanan 2005) can better approximate perfection.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Appendix S1. Details of methods and statistical analyses for data in Box 3.

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